Effects of *In Ovo* Herbicide Exposure in Newly Hatched Domestic Chickens (*Gallus gallus*) and Ducks (*Anas platyrhynchos*)

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by

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PREFACE

This thesis has been organized as a series of manuscripts that will be submitted for publication in scientific journals. Some repetition of introductory and methodological material is unavoidable.

ABSTRACT

Agriculture is a valuable economic resource in western Canada, but for decades farmers have focused on intensive production practices while ignoring the long-term health and maintenance of the land. In recent years, the use of conservation agricultural techniques has been encouraged in an effort to conserve prairie landscape while sustaining cropland productivity. Sustainable agricultural practices that promote soil and water conservation and benefit wildlife and prairie biodiversity include conservation tillage and planting of winter cereal crops. Many species of wild birds nest in the ground cover provided by minimum tillage and fall seeded cropland in the spring. Although habitat quality in conservation areas is superior for birds, there is potential for eggs of ground nesting birds to be exposed to herbicides during spring weed control operations. Herbicides commonly used on the prairies to control weed growth in conservational systems include 2,4-D and Buctril-M®. Since the subtlethal effects of exposure to these herbicides may include DNA damage and immunomodulation, the overall goal of this study was to assess whether *in ovo* exposure to the herbicides 2,4-D and Buctril-M® adversely affects genetic material and/or immune system function in newly hatched domestic chickens (*Gallus gallus*) and ducks (*Anas platyrhynchos*), as surrogates for wild bird species.

Study design attempted to reproduce actual field exposures by use of an agricultural field spray simulator to apply formulated herbicides (as opposed to pure active ingredients) at recommended crop application rates. In three separate experiments, fertile chicken eggs were sprayed with 2,4-D ester formulation or with Buctril-M® formulation, and fertile duck eggs were sprayed with 2,4-D ester formulation, during either an early (embryonic day 6) or late (embryonic day 15 for chickens or embryonic day 21 for ducks) stage of incubation. Genotoxicity and immune system function were evaluated in the hatchlings as the main toxicological endpoints to assess potential subtle effects from herbicide exposure, but additional measures of general health and development were also evaluated. Two endpoints were used to assess subtle changes to genetic integrity. The comet assay was used to detect structural damage (strand breaks) in avian lymphocyte DNA, as an index of acute genotoxic effects. Flow cytometry was used to examine potential clastogenic effects of the herbicides,

by determining if chromosomal changes resulted in variability in the DNA content of avian erythrocytes. Several endpoints were examined to evaluate potential exposure-induced effects on the immune system. Immunopathological assessment of chicks and ducklings included differential lymphocyte counts, as well as immune organ weights and histopathology. The cell-mediated and humoral immune responses in hatchlings were assessed using the delayed-type hypersensitivity test and measurement of systemic antibody production in response to immunization, respectively.

Exposure of fertile chicken and duck eggs to Buctril-M® or 2,4-D had no effects on the biomarkers of genetic integrity in this study. Differences in herbicide treatment (high and low concentrations) and times of exposure (early and late incubation stages) did not translate into noticeable factor effects in final model analyses for any of the genotoxicity assay variables evaluated in newly hatched chickens exposed *in ovo* to 2,4-D. Similarly, comet assay outcomes in chicks exposed to Buctril-M® were not significantly associated with either herbicide treatment or time of exposure as fixed effect factors. Results of the comet assay using peripheral lymphocytes from ducklings provided evidence of potential primary genetic damage associated with the time of spray exposure *in ovo*. Comet tail DNA content was significantly associated ($P = 0.03$) with exposure times, suggesting that ducks may be increasingly sensitive to spray exposure conditions at an early stage of embryological development. Effects of exposure timing were not attributable to herbicide treatment. Although 2,4-D exposure time was associated with DNA strand breakage in ducklings, there was no evidence of chromosomal damage. However, an association between the HPCV values (a measure of DNA content variability) and time of spray exposure was observed in the experiment where 21-day-old chickens were treated *in ovo* with Buctril-M®. The mean HPCV value for the early exposure group (E6) was significantly greater ($P = 0.02$) than that of the group treated later in incubation (E15). However, Buctril-M® the concentration of herbicide did not have any influence on this outcome, and the reason for the difference between exposure times is uncertain, but may be attributed to stress associated with manipulations during spraying. An increase in HPCV, reflecting greater intercellular DNA variability, is indicative of increased incidence of chromosomal damage, which may be an effect of disturbance during early periods of incubation as a result of exposure conditions.

Among the panel of immunotoxicity tests conducted to evaluate the effects of *in ovo* exposure to 2,4-D and Buctril-M® on the developing avian immune system, only heterophil/ lymphocyte (H/L) ratios and relative immune organ weights were significantly associated with either herbicide treatment or time of spray exposure in all three experiments. In 21-day-old chicks exposed *in ovo* to 2,4-D, relative bursa weight was associated with the different herbicide treatments ($P = 0.0006$). Relative bursa weights were significantly lower in chicks in the low dose group, while the opposite effect was observed in the high dose chicks, compared with the controls. It is unlikely that the observed decrease in bursa weight in the low dose group is causally related to herbicide exposure because a consistent dose-response effect was not observed, but this outcome may be explained by a compensatory immune response. The relative spleen weights of newly hatched chickens exposed *in ovo* to Buctril-M® exhibited a significant association with herbicide treatment ($P = 0.01$). Relative spleen weights for birds in the low dose treatment groups were significantly different than both the control ($P = 0.02$) and high dose groups ($P = 0.01$). However, there was no significant difference between high dose and control groups, and this outcome reduces the likelihood of a causal relationship between spleen weight and herbicide exposure. In the parallel experiment involving *in ovo* exposure to 2,4-D to ducklings, relative bursa weight was associated with time of spray exposure ($P = 0.04$). Ducklings that hatched from eggs exposed to spray on day 6 of incubation exhibited greater mean relative bursa weights than the birds exposed to spray at a later incubation stage (E21). This result implies that spray exposure during earlier stages of development may result in conditions which affect the humoral immune response, if increased bursal weight is associated with increased B lymphocyte and antibody production. In the same experiment, mean H/L ratios in peripheral blood samples from 21-day-old ducklings were significantly different between the groups treated with the high concentration of 2,4-D and water (control) ($P = 0.04$). Although ratios from the birds in the low dose groups were not significantly different from the control groups, changes in H/L ratio values demonstrate a dose dependent relationship with increasing herbicide exposure.

Residue analysis of chicken and duck eggs in this study measured transfer of herbicide through the shell and into the embryo 24 hours and up to 5 days (chickens only) after spraying. Mean 2,4-D residue concentrations were higher in both chicken and duck eggs

from the high dose (10X) groups than in eggs exposed to the recommended field rate of herbicide application (1X). Embryo residue concentrations in the chicken eggs increased from the day following exposure to 5 days after spraying, in both low and high dose groups. This observation indicates that the risk of contaminant-induced adverse effects may continue to increase for at least several days after exposure, thereby influencing the concentration of herbicide to which the developing embryo is exposed.

On the Canadian prairies, wild bird eggs are potentially to be exposed to 2,4-D and Buctril-M® during various stages of embryonic development. The present study examined effects of herbicide exposure at two distinct times during incubation, and demonstrated the potential for subtle impacts on genetic integrity and the immune system. Results indicate that spray exposure during earlier stages of organogenesis may cause more significant adverse effects. Given the possible harmful consequences of the observed changes on the long-term health of wild birds, further research is needed in order to better characterize the risks of *in ovo* agrochemical exposure in prairie ecosystems.

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DEDICATION

This thesis is dedicated to my family...

Mom, Dad, Kelly & Leigh,

I love you always.

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2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
APC	antigen presenting cell
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CMI	cell mediated immunity
CV	coefficient of variation
DMSO	dimethyl sulfoxide
DNP-KLH	dinitrophenol-keyhole limpet hemocyanin
DTH	delayed-type hypersensitivity
DU	Ducks Unlimited
EDTA	Ethylenediaminetetraacetic Acid
ELISA	enzyme-linked immunosorbent assay
H/L	heterophil/lymphocyte
HPCV	half-peak coefficient of variation
Ig	Immunoglobulin
LC	liquid chromatograph
MS	mass spectrometry
MCPA	4-chloro-2-methylphenoxyacetic acid
MHC	major histocompatibility complex
NK	natural killer
PCB	polychlorinated biphenyl
PBS	phosphate buffered saline
PI	propidium iodide
PP	peroxisome proliferator
SCE	sister chromatid exchange
SRBC	sheep red blood cell
WBC	white blood cell

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Project Background

Agriculture is the dominant land use and a major economic force on the Canadian prairies, with millions of tonnes of crops produced annually. However, certain agricultural practices are environmental stressors, resulting in significant changes to the prairie landscape, and to wildlife habitat and biodiversity. Major impacts of agriculture on the Canadian prairies include soil erosion, wetland drainage, water diversion and contamination, and air quality issues (Agriculture & Agri-Food Canada [AAFC] and Saskatchewan Agriculture, Food and Rural Revitilization [SAFRR] 2004). The agricultural sector in Canada recognizes that in order to maintain production and ensure the future vitality of the industry, farmers must adopt practices that minimize impacts on the natural environment (AAFC 2003). Over the last few decades, intense efforts have been made to develop and implement conservation techniques that protect and maintain the usability of environmental resources. Topsoil loss has been reduced by application of minimum tillage techniques, and direct seeding into the previous year's crop stubble. Low till methods are also practiced during winter crop planting, a strategy which is increasingly used by farmers for conservation and other benefits (Phillips et al. 1980, Gebhardt et al. 1985). Adoption of soil management practices not only minimizes erosion rates, but also helps to maintain water and air quality, and conserve wildlife and prairie biodiversity.

Farms that employ soil conservation techniques such as minimum till and winter crop planting typically support greater wildlife use than conventionally tilled farmland (Castrale 1985, Wooley et al. 1985). In no-till fields the ground remains idle throughout the fall and spring seasons, and crop residue remains undisturbed, persisting on the soil year-round. In winter cropping systems, the previous year's crop stubble is left in the soil to provide protection to over-wintering seeds. Crop residue left by both agricultural techniques may increase available habitat for small mammals and ground-nesting birds. Tillage systems

in particular, affect birds that breed in cropland by influencing the residue available for nesting cover. Several studies have shown that bird usage and nest densities are generally higher on untilled upland habitats as opposed to fields that receive tillage (Higgins 1977, Basore et al. 1986, Lokemoen and Beiser 1997).

While the potential of conservation tillage to improve the fortunes of prairie-nesting birds is great, the health and productivity of native birds may be affected by agricultural practices other than cultivation. Pesticide application remains an important part of crop production in minimally tilled fields, and in those used to grow winter cereals (Gebhardt et al. 1985, Campbell 1999). Conventional tillage effectively controls the majority of persistent weed growth in cropland. However, with the adoption of conservation tillage practices (less mechanical weed removal), cultivation operations are often replaced with intensified herbicide application to control weeds (Gebhardt 1985). The potential impact on wildlife species of this increased reliance on chemical weed control is a concern to farmers and wildlife managers. Because the timing of herbicide application often coincides with the nesting period of many species of birds, eggs of ground nesting species are at risk of exposure (Castrale 1985, Wooley et al. 1985).

Herbicides are generally much less acutely toxic to terrestrial vertebrates than insecticides, but relatively little is known about the potential sublethal effects of exposure to these chemicals on wild species; especially when exposure occurs during embryonic development. Consequently, in order to confirm the environmental sustainability of conservation tillage practices for wildlife, it is important to investigate potential adverse effects of increased exposure to commonly used pesticides associated with these practices.

Sublethal effects of environmental contaminants on wildlife species are frequently evaluated using controlled studies with surrogate species that employ sensitive measures of exposure (effects biomarkers). Structural changes to DNA and modulation of the immune system are examples of useful biomarkers for assessing sublethal impacts of exposure to environmental pollutants. Studying biomarkers of genetic and immune system alteration may reveal the potential for subsequent health effects at individual or population levels (Dietart and Golemboski 1994, Kleinjans and van Schooten 2002). The intent of this project is to evaluate subtle changes in genetic integrity (genotoxic effects) and immune function (immunotoxic effects) associated with *in ovo* exposure of bird embryos to commercial

herbicides commonly used on the Canadian prairies. Domestic chickens (*Gallus gallus*) and ducks (*Anas platyrhynchos*) were selected as surrogates for wild species of upland game birds and waterfowl, respectively.

1.2 Sustainable Agricultural Practices: Conservation Tillage and Winter Cereal Crops

Advances in Canadian agriculture in recent years have generally focused on improving crop production through development of fertilizers to mitigate soil nutrient deficiencies, pesticides to control crop pests (plant and insect), improved seed varieties, and better seeding and tillage techniques. Implementation of all of the above has resulted in increased crop yield and significant economic gain (Lafond and Fowler 1990). The latest phase of agricultural research has shifted focus to the development and application of production systems that emphasize soil and water conservation. These new technologies and practices are required to address such prairie agri-environmental issues as topsoil erosion, soil organic matter decline, soil water depletion, and surface water contamination (AAFC and SAFRR 2004).

A popular phrase used to describe this conservation movement is "environmentally sustainable agriculture" (Strang 2004). "Sustainability implies that we pay increasing attention to the long-term effects of our agricultural practices and place a greater importance upon health and environmental issues than we have in the past" (Wright 1990). Conserving topsoil and improving the health of soil resources are crucial aspects of sustainable agriculture. In 1995, Agriculture & Agri-Food Canada identified specific agricultural conservation techniques that have contributed to reducing the risks of soil erosion. Of these, conservation tillage was identified as an important method to prevent topsoil loss (Acton and Gregorich 1995).

Traditional tillage methods are responsible for many negative environmental effects of agriculture. The effects of tillage on soil processes are particularly substantial, and include increased soil erosion, runoff, and desiccation. Conventional tillage leaves the ground uncovered so that any natural weathering processes will easily detach, transport, and re-deposit soil particles (Gebhardt et al. 1985). Every time the soil's surface is disturbed by tillage, valuable moisture is lost through evaporation (Campbell 1999). Tilling soil also makes soil particles susceptible to increased wind erosion, such that the combined effects of wind and water erosion have resulted in cropland degradation and significant farm income

loss. More than 20 years ago, an evaluation of Canadian land resources estimated that losses from soil degradation on the prairies exceeded $1 billion annually (Science Council of Canada 1986). The potential for individual loss of revenue is one of the factors that has encouraged farmers to adopt conservation agricultural techniques in recent years.

Soil erosion is virtually eliminated when no-tillage agriculture is practiced. In general, the rate of topsoil loss increases with tillage frequency and decreases as amounts of residues or plant cover increase (Phillips et al. 1980). In no-till cropland, the ground remains idle throughout the fall and spring, allowing plant residue to persist on the soil year-round. Under conservation tillage, residue from previous crops acts as a buffer to soil weathering processes, and erosion is limited (Gebhardt et al. 1985). No- and low-till farming practices are rapidly gaining acceptance in Canada, especially in the prairie provinces. According to the latest agricultural census by Statistics Canada, almost 63% of all land prepared for seeding (cropland) in the prairies received minimal till treatment, with 61% of Prairie farmers practicing zero-till techniques (Statistics Canada 2001, Boame 2005).

Winter cereal planting has also become a popular agricultural strategy, in part for its conservation benefits (AAFC and SAFRR 2004, AAFC 2005). When crops are grown in fields with no-till residue, as during direct seeding of winter cereals, the remaining crop stubble serves to protect the over-wintering seed, increasing the benefits of both practices. In 2003 and 2004, winter wheat saw near-record production in the prairie provinces, with approximately 700,000 seeded acres (Western Grains Research Foundation, 2005).

1.3 Agriculture and Wildlife: Avian Use of Conservation Fields

Aside from promoting soil and water conservation, reduced tillage practices and winter cereal planting also benefit wildlife and prairie biodiversity. For example, research has shown that farms employing these conservation techniques support richer avifauna than conventional farms (Shutler et al. 2000). Several authors (Higgins 1977, Castrale et al. 1985, Basore et al. 1986) have studied the responses of wild birds to various tillage conditions, and concluded that cropland that received reduced tillage was more attractive to nesting birds than conventionally tilled fields. Higher use of these areas by wildlife has been attributed to increased ground cover in the spring, with many species of birds nesting in the stubble provided by minimum tillage, and in early emerging winter cereals (Lokemoen and Beiser 1997). The fact that fall planted crops remain undisturbed by tillage during the April-July

nesting season led Ducks Unlimited (DU) Canada to promote fall planting as a means of enhancing productive nesting habitat for waterfowl. As early as 1983, DU Canada began promoting winter cereal crops that featured zero till seeding and few or no field operations during the spring nesting season to benefit breeding waterfowl in the prairie pothole region (Cowan 1985). More recently, research conducted by DU Canada and a collaborative conservation cover program ("Conserve and Win Program") with the University of Saskatchewan provided additional support for the planting of winter wheat, and acknowledged the benefits of additional ground cover to encourage nesting of waterfowl in prairie cropland (Fowler 2002, Devries and Moats 2005).

1.4 Herbicide Use and Potential *In Ovo* Avian Exposure

Although habitat quality for wildlife species, especially birds, is superior in reduced tillage fields compared with conventional cropland, the need for increased herbicide application in conservation systems is a potential concern. Changes in crop management systems may result in a difference in weed populations. A disadvantage of minimal tillage practices is the increase in application rates of herbicides usually required to maintain adequate weed control (Castrale 1985, Holm 1990). Wildlife use of conservation tillage fields creates a potential for increased contact with agricultural chemicals. Herbicide treatment usually occurs from mid-May until mid-June on the Canadian prairies, a time coinciding with the nesting period for many birds. Consequently, the eggs of ground nesting species are at risk of being exposed. Possible routes of egg exposure to herbicides include transfer of chemical residues from contaminated feathers of incubating parents, or direct spraying of eggs and young birds in the nest (Wooley et al. 1985, Hoffman 2001). It is highly probable that large numbers of waterfowl and upland game bird eggs are directly exposed to herbicide spray every year if the incubating parent is absent at the time of application, or is flushed from the nest in response to the disturbance from approaching sprayer machinery. Ground nesting species most at risk on the Canadian prairies probably include upland game birds such as the Ring-necked pheasant (*Phasianus colchicus*) and the Hungarian pheasant (*Perdix perdix*), and various waterfowl, including mallard (*Anas platyrhynchos*) and northern pintail (*Anas acuta*) ducks and certain goose species. This increase in potential exposure during the vulnerable period of embryonic development makes

it important to investigate the possible adverse effects of herbicides commonly used on winter wheat or no till cropland that is particularly attractive to ground nesting birds.

2,4-Dichlorophenoxyacetic acid (2,4-D) is the world's most widely used herbicide. It is estimated that more than 3.8 million kilograms of 2,4-D is applied annually (Environment Canada 2001). Popular for its selective properties, 2,4-D is a chlorophenoxy herbicide that targets broadleaf and woody plants, and it is the predominant herbicide used for weed control in winter wheat, especially in the spring (Fowler 2002). The herbicide formulation Buctril-M®, containing a 50:50 mixture of bromoxynil (4-cyano-2,6-dibromophenol) and 4-chloro-2-methylphenoxyacetic acid (MCPA), is also frequently used on the prairies for weed control in winter wheat, and along with 2,4-D, is one of the top herbicides applied to this type of crop (Fowler 2002). Since the popularity of minimally tilled fields is growing, and 2,4-D and Buctril-M® are widely used in spring seeded crops, it is likely that other low-till areas receive similar herbicide treatment. 2,4-Dichlorophenoxyacetic acid is available in a number of different commercial formulations, and along with bromoxynil and MCPA, is approved for use in tank mixes with numerous other herbicides. Therefore, the potential for wildlife exposure to one of these agents is significant.

1.5 Potential Toxicity of 2,4-D and Buctril-M® to Birds

Although herbicides are designed to kill plants, they can be toxic to mammals and birds as well. Relatively little is known about the potential long-term effects of low rates of exposure to these chemicals in wild species, especially when exposure occurs during embryonic development. There is evidence to suggest that, in addition to the active ingredients in herbicide formulations, commonly used surfactants and/or emulsifiers may also contribute to potential wildlife toxicity. Adjuvants are added to pesticide mixtures to enhance the effectiveness of the active components, but these additional chemicals may enhance pesticide toxicity by changing the toxicokinetic properties (absorption, distribution, etc.) of the active ingredients (Lin and Garry 2000). Therefore, studies of herbicide toxicity to wildlife should include evaluation of commercial herbicide formulations as used in the field, rather than pure active ingredients.

Structural changes to DNA and immune modulation are useful and increasingly common bioindicators for assessing the sublethal toxicity of contaminants in wildlife species (Dietart and Golemboski 1994, Kleinjans and van Schooten 2002), including animals

exposed during embryonic development. Damage to DNA (chromosomal aberrations, micronuclei, and strand breaks) can be a precursor to permanent, significant genetic changes, including alterations associated with carcinogenesis or developmental defects (Ponder 2001). Therefore, contaminant-induced changes in biomarkers of DNA damage may be indicative of potential population-level genotoxic effects that may impact fitness (Kleinjans and van Schooten 2002).

The genotoxic potential of 2,4-D is uncertain. It has been studied in a number of test systems with conflicting results that range from high chromosomal damage to none at all. Inconsistent results may be due to the use of different chemical formulations of 2,4-D, different test systems, and/or analysis of different genotoxic endpoints. Mutagenic, clastogenic, and genotoxic effects have been observed in tests using both mammalian and non-mammalian cells (Venkov et al. 2000, Amer and Aly 2001, Ateeq et al. 2005, González et al. 2005). Both 2,4-D and MCPA have been reported to cause peroxisome proliferation in mammalian cells (Vainio et al. 1982). Peroxisome proliferators are generally referred to as non-genotoxic carcinogens, but some peroxisome proliferators cause induction of sister chromatid exchange, chromosomal aberrations, and micronuclei (Arias 1992 & 1996, Dzhekova-Stojkova et al. 2001, Madrigal-Bujaidar et al. 2001, Arias 2003). Consistent with this potential mechanism, 2,4-D and MCPA have been shown to induce chromatid exchange, clastogenicity and micronuclei in mammalian and avian cells in both *in vitro* and *in vivo* experiments (Korte and Jalal 1982, Turkula and Jalal 1985, Schop et al. 1990, Arias 1992, Madrigal-Bujaidar 2001, González et al. 2005).

The sublethal toxic effects of bromoxynil are not as well known as the phenoxy herbicides. However, in acute toxicity tests, bromoxynil is highly to moderately toxic to many avian species, including pheasants, hens, quail, and mallard ducks, with an LC_{50} as low as 50 mg/kg (Kidd and James 1991). It has also been shown to affect development in rats, and is a suspected teratogen (Rogers et al. 1991, Chernoff et al. 1991). Although bromoxynil has not been shown to be genotoxic or carcinogenic, sublethal effects on bird embryos may occur.

Alterations to the normal functioning of the immune system may result in either a reduction or enhancement of the immune response (immunomodulation). Many environmental contaminants, including some herbicides, are known to cause immune

dysfunction (Loose et al. 1978, Colosio et al. 1999). Several studies have demonstrated that 2,4-D has the potential to affect the immune system. For example, it was shown that 2,4-D treatment in mice caused a reduction in the amount of antigen-specific antibody-secreting B cells produced in the bone marrow, resulting in a decrease of serum antibody titres (Salazar et al. 2005). The *n*-butylester of 2,4-D was found to differentially affect antibody production in mice, depending on the route of exposure (Blakley 1986, Blakley and Schiefer 1986). Exposures to herbicide mixtures containing 2,4-D as a main component also have immunotoxic effects. Tordon 202C exposure resulted in a decrease in the T-lymphocyte-dependent primary humoral (antibody) immune response in mice (Blakley 1997). Thymocyte depletion in association with thymic atrophy was also observed in mice dosed with 2,4-D and propanil (3,4-dichloropropionanilide) (de la Rosa et al. 2005). Alterations in immune response suggestive of immunosuppression have been observed in humans chronically exposed to phenoxy herbicides. A significant reduction in peripheral blood T-cell and natural killer cell populations, as well as a decreased T-cell proliferative response to mitogen stimulations were reported in people exposed to commercial 2,4-D and MCPA formulations (Faustini et al. 1996). 4-Chloro-2-methylphenoxyacetic acid has also been shown to be immunotoxic, affecting lymphocyte activation in sheep (Pistl et al. 2003). Although no studies have linked bromoxynil to negative effects on the immune system, association with MCPA in the Buctril-M® formulation may cause detectable changes in immune system function. Consequences of chemical-induced immune alterations may vary from slight changes in immune responses without any indication of health impairment, to significant immunosuppression leading to altered host resistance and increased susceptibility to infectious diseases that may impair fitness (Fairbrother et al. 2004). Therefore, evaluation of potential sublethal effects of *in ovo* exposure of birds to 2,4-D and Buctril-M® should include assessment of immune function.

1.6 Genotoxicity Assessment

1.6.1 The Comet Assay: Measurement of DNA Strand Breaks

The comet assay, also known as the single-cell gel electrophoresis assay, is a rapid and sensitive method for the detection and visualization of DNA damage (single and double strand breaks and alkali-labile DNA sites) in individual cells (Sutherland and Costa 1999, Collins 2004). DNA strand breakage may occur under normal physiological conditions, but

exposure to genotoxicants has been shown to increase the occurrence and frequency of breaks (Shugart 1994). The comet assay can be applied to virtually every type of nucleated eukaryotic cell exposed *in vitro* or *in vivo* to a variety of physical or chemical agents (McNamee et al. 2000). Because the assay measures damage in individual cells, it can provide information on intercellular variability, and assess similar effects in all cells within a population.

Assays that measure strand breaks are generally based on the principle that the lesions reduce the size of the large duplex DNA molecule, and single and double-strand breaking agents can have dramatic effects on the supercoiled chromatin within the nucleus. In the comet assay, a high pH (~13) is used to facilitate cellular denaturation and DNA unwinding, and subsequent identification via electrophoresis of existing single and double-strand breaks, as well as breaks that only become apparent after exposure to alkali conditions (alkali-labile sites) (Fairbairn et al. 1995). Individual cells are embedded in a gel and subjected to an electrophoretic field, followed by visualization of DNA using a fluorescent dye. After electrophoresis, cells with DNA strand breaks have the appearance of a comet, with a head (the nuclear region) and discernible tail (consisting of negatively charged DNA fragments or strands that migrate away from the nucleus toward the anode) (McKelvey-Martin et al. 1993, Hartmann et al. 2003).

The amount of damaged DNA is expressed by measuring the size and fluorescent intensity of the comet tail. The intensity of the stain in the comet's tail region is related to DNA content, and DNA damage can be estimated from measurements of the amount of DNA in the tail. The size of the tail, a function of fragment size and migration distance of DNA strand breaks (Fairbairn et al. 1995), is an additional description of genetic effects. Sensitivity of damage detection will depend on the assay methodology, as well as the variables used to measure the comet formation. The alkaline version of the comet assay was developed to increase sensitivity for detection of low levels of genetic damage, and comet tail intensity and size are the most common measurements currently used to quantify these changes (Singh et al. 1988).

The revised alkaline method of the comet assay was introduced in 1988 (Singh et al.). Because of its relative simplicity and sensitivity, it has been used as a biomonitoring technique in (eco)genotoxic evaluation of numerous environmental contaminants. Reviews

of the literature reveal a multitude of applications, including: plants, invertebrates (earthworms, mussels, oysters), amphibians, fish, mammals (Fairbairn et al. 1995, Cotelle and Ferard 1999, Ateeq et al. 2005, González et al. 2005), and birds (Maness and Emslie 2001, Pastor et al. 2001a & 2001b, Dušek et al. 2003). The model of the chick embryo exposed to environmental contaminants *in ovo* is ideal for genotoxicity studies of avian species. However, prior to the present study, very few researchers have used this exposure technique in conjunction with the comet assay to evaluate DNA damage in developing birds (Dušek et al. 2003).

1.6.2 Flow Cytometry: Measurement of DNA Variability

The use of flow cytometry to measure variability in DNA content among cells is a recognized method in genotoxicity assessment. Increased variability of nuclear DNA content in a specific cell population of an organism is considered indicative of irreversible (and potentially heritable) chromosomal damage (Otto and Oldiges 1980, Deaven 1982, Shugart 1994). The flow cytometric method has previously been used to document the clastogenic effects of many physical and chemical contaminants, including pesticides (Bickham et al. 1988, George et al. 1991, Bickham et al. 1992, Bickham et al. 1994, Custer et al. 1994, Lamb et al. 1995, Lowcock et al. 1997, Custer et al. 2000, Matson et al. 2004). Clastogenic agents, by definition, have the ability to induce structural alterations in chromosomes, with consequent effects ranging from mutations and chromosome breakage to improper chromosome reattachment and reformations. These alterations may result in an unequal allocation of nuclear DNA to daughter cells following cell division (Lamb et al. 1995, Misra and Easton 1999). Dispersion of DNA content in a population of cells is described using the coefficient of variation (CV) or half-peak CV (HPCV) of a sample of cells in the resting (G_1) phase. An increase in CV or HPCV, demonstrating greater variability in intercellular DNA content, is indicative of increased incidence of chromosomal damage (Otto and Oldiges 1980). Several studies using simultaneous evaluation of standard karyology and flow cytometry have confirmed the relationship between chromosome breaks and increases in the CV of DNA content (McBee et al. 1987, McBee and Bickham 1988, Bickham 1990).

Using flow cytometry to measure clastogenic effects is based on the principle that fluorescent emissions from cells stained with DNA-binding fluorochromes can be measured and positively associated with DNA content. With this technique, individual cellular DNA

content is estimated as stained cells are passed through a flow system as a stream of single cells. The cells encounter a laser excitation beam which excites the fluorescent dye, and the visible light emitted is measured by a photometer (Bickham 1990). Flow cytometry is particularly sensitive in detecting effects of environmental mutagens that induce clastogenic changes. Advantages of this technique over other chromosomal assays include the ease and simplicity of sample-cytometer introduction, rapid and inexpensive analysis of a large number of cells of many different tissue types, and (in the case of blood cells), the potential for easy, repeat, nondestructive access to cell populations from virtually any organism (Deaven 1982, Bickham 1990). In many respects, flow cytometry is an ideal method to assess genotoxicity in ecological studies.

1.7 Immunotoxicity Assessment

Assessing the immune response and potential immunomodulating effects of environmental contaminants has become an important approach to assessing sublethal toxicity in wildlife species. The immune system is dynamic and interactive, with continual cellular development and differentiation, making it especially vulnerable to toxic insult (Dietart and Golemboski 1994, Keller et al. 2000). Substances that interfere with the development, structure or function of the immune system are deemed immunotoxic. Evaluation of contaminant induced changes in the immune system provides valuable information about potential adverse effects on wildlife health, and may be applicable in assessing risks to human health (Colosio et al. 1999, Keller et al. 2000, Grasman 2002, Fairbrother et al. 2004).

1.7.1 Overview of the Avian Immune System

Current knowledge of the avian immune system is largely based on domestic fowl and poultry research. Assuming that close similarities exist between the poultry immune system and that of other avian species, studies with domestic chickens have contributed greatly to our understanding of the fundamental concepts of avian immunology (Vainio and Imhof 1995, Glick 2000, Fairbrother et al. 2004). As in mammals, the avian immune system functions to defend the host against potentially harmful foreign substances, providing protection against “nonself” components including microbial pathogens, parasites, unfamiliar proteins, and neoplastic cells (Fairbrother et al. 2004). In healthy animals, all parts of the immune system, working together, are capable of defending the host against most foreign

substances. However, if immune system function is compromised by immunotoxicant exposure (or other stressors), host resistance may be impaired and health may be threatened (Loose et al. 1978, Keller et al. 2000). It is important then, to understand the typical functions and responses of the avian immune system in order to recognize potentially harmful changes.

Immune organs are collectively named "lymphoid" because they support the production and/or function of lymphocytes, the functional components of the specific or adaptive immune system. The cellular population of the avian innate immune system is comprised of T and B lymphocytes, natural killer (NK) cells, and phagocytic cells (macrophages and heterophils) (Fairbrother et al. 2004). Primary lymphoid structures of the avian immune system include the bursa of Fabricius, thymus, and bone marrow. The bursa and bone marrow are the sites of B lymphocyte production in birds, while the thymus is the site of T lymphocyte development. Secondary lymphoid structures include the spleen, lymph nodes, and various lymphoid tissues such as those associated with the mucosa of the gut, respiratory tract, and eyes (Glick 2000, Keller et al. 2000, Fairbrother et al. 2004). Research with domestic chickens and chick embryos first described the major role of lymphocytes in cellular immune responses (Davison 2003).

There are two different, yet interactive, categories of typical immune system responses: innate (non-specific) and acquired (specific). The innate immune response is characterized by phagocytosis and destruction of foreign agents by macrophages, neutrophils, eosinophils, basophils, etc. This non-specific response lacks any immunological memory, but is immediate, and represents the first line of defense against invaders that get through physical barriers, such as the skin and mucous membranes. Acquired immunity is different from innate in that the response is specific (for a given foreign agent) and has memory. This type of immunity develops slowly, but responds rapidly if the same invader is encountered again. Acquired immunity is further subdivided into cell-mediated and humoral responses (Larsson and Carlander 2002, Fairbrother et al. 2004).

The humoral immune response is mediated by B cells which produce antibodies, which are protective glycoprotein molecules with specific receptors for binding foreign antigens (Larsson and Carlander 2002). Antibodies are produced by differentiated B cells which originated in the bursa of Fabricius in birds (bone marrow in mammals) (Mathew et al.

2002). Antibodies are found in the serum or mucosal secretions of sensitized animals. Different types of antibodies are produced depending on function, location within the body, and time course of infection. Birds are currently thought to have only three antibody classes: immunoglobulin (Ig) M (primary antibody), IgY (secondary response antibody, equivalent to mammalian IgG), and IgA (mucosal antibody). Immunoglobulin Y is the main serum antibody in birds (Benedict and Berestecky 1987).

A humoral response is stimulated by exposure to foreign antigen, and culminates in the production of antibodies that specifically bind and destroy the antigen. Initial exposure results in a primary response to the antigen, while repeat exposure triggers a secondary response. These responses differ both qualitatively and quantitatively, with the secondary response usually being more rapid, persistent, and characterized by higher serum antibody concentrations than the primary response. The initial response is generated by naïve B cells encountering the antigen for the first time. Activated B cells differentiate into plasma cells that secrete IgM class antibodies specific to the antigen (Mathew et al. 2002). A secondary response involves proliferation of B cells that retained the memory of the primary event, resulting in the production of large amounts of antigen-specific antibodies (IgG/IgY(avian)) (Fairbrother et al. 2004).

The B cell response to T cell dependent antigens requires T helper cells first recognizing antigens associated with cell surface glycoproteins encoded by the major histocompatibility complex (MHC) (Fairbother et al. 2004). MHC derived molecules consist of three major classes in mammals, and a fourth class in chickens. Class I are surface proteins co-expressed with endogenous peptides on virtually all nucleated cells in the body, and erythrocytes. Class II proteins coexist with peptides expressed in B cells, plasma cells, macrophages and activated T cells, and aid with T helper cell antigen recognition. Class III MHC encodes for several complement proteins, while class IV proteins are expressed solely on B and T cells, erythrocytes and thrombocytes in chickens (Larsson and Carlander 2002).

The cell mediated immune response (CMI) is characterized by the action of T lymphocytes to identify and eliminate foreign antigenic molecules. Initiation of CMI involves interaction of the membrane T cell receptor and the foreign antigen through recognition of specific peptide fragments displayed by antigen presenting cells (APCs). These cells present the antigen fragments bound together with cell surface molecules

encoded by genes of the MHC. Antigen recognition by naïve T cells stimulates their proliferation and differentiation into effector and memory cells.

Effector T cells recognize antigens bound to APCs, and are stimulated to eliminate the antigen through two possible pathways. Effector T cells can act either to enhance the immune response (helper cells, with CD4+ surface antigens) or suppress the response (suppressor T cells, which secrete inhibitory cytokines) (Larsson and Carlander 2002, Tizard 2004). Effector T cells of the CD4+ subset express membrane molecules and secrete cytokines that attract and activate macrophages to eradicate the antigen through phagocytosis. CD8+ cytolytic T cells kill infected cells that display class I MHC-associated antigens. Memory T lymphocytes recognize antigen upon repeat exposure. These cells are able to respond quickly to subsequent encounter with the antigen and differentiate into effector cells that eliminate the antigen (Abbas 2005).

1.7.2 Immunotoxicity Assays

An accepted method of assessing the effect of xenobiotics on the immune system is to examine the characteristics of associated cells, tissues, and organs in exposed animals. Sampling and studying these components, termed immunopathology, provides general information about immune structure and function (Keller et al. 2000). Methods developed for immunotoxicity assessment have been organized into a tiered screening system by the National Toxicology Program in the USA. The Tier I screen involves initial assessment of immunotoxic effects using functional assays which evaluate cell-mediated and humoral immune responses, as well as histopathology of immune organs. Tier I comprises tests that are relatively simple and inexpensive to perform, and includes complete and differential blood cell counts, immune organ weights and histology, and simplified functional assays for cell-mediated and humoral immunity, such as graft rejection response tests and the sheep red blood cell (SRBC) hemagglutination assay, respectively (Weeks et al. 1992, Schuurman et al. 1994).

Immune function assays in Tier II of the screening process offer more in depth analysis of all components of the immune response. Tier II is comprised of comprehensive tests to further define an immunotoxic effect. If a compound appears to be immunotoxic in Tier II assays, it would be expected to have demonstrated some effect(s) in Tier I tests. Therefore, Tier II tests are used to identify the mechanism of action of immunotoxicants

through specific tests for cell-mediated immunity, secondary antibody responses, lymphocyte quantification, and host resistance models. Application of as few as two or three immune function tests is sufficient to identify immunotoxic compounds in rodents (Luster et al. 1992, Luster et al. 1993). The present study employed several Tier I and Tier II immunotoxicity tests to evaluate potential immunomodulating effects of herbicides in developing birds.

1.7.2.1 Humoral Immune Response Assessment

The humoral immune system can be evaluated by measuring B lymphocyte activation following antigen exposure (Abbas 2005). The enzyme-linked immunosorbent assay (ELISA) is a common technique to measure the amount of antigen-specific antibody present in a blood serum sample following exposure to an antigen, and is a sensitive means of evaluating the ability to mount a humoral immune response (Margulies 2000, Smits and Janz 2005).

Numerous ecotoxicological studies have used the ELISA technique to evaluate the humoral immune function of wild species exposed to environmental contaminants (Smits et al. 1996, Sanchez-Dardon et al. 1999, Bunn et al. 2000, Regala et al. 2001, Beckmen et al. 2003, Gilbertson et al. 2003, Sures and Knopf 2004). In recent years, there has been increasing use of the ELISA to detect specific immunoglobulin levels in wild birds. For example, Bustnes et al. (2004) reported a decreased antibody response to immunization with diphtheria toxoid in female glaucous gulls (*Larus hyperboreus*) with high blood concentrations of organochlorine pesticides (hexachlorobenzene or oxychlordane). The ELISA has been used to measure antibody responses in western bluebird (*Sialia mexicana*) nestlings exposed to lead shot (Fair and Myers 2002) and in American kestrels (*Falco sparverius*) exposed to polychlorinated biphenyls (Smits and Bortolotti 2001). This assay is also commonly used to measure humoral immune function when the overall health or condition of wild bird populations is questioned. It has been used to measure antibody responsiveness in blue tits (*Parus caerulues*) to evaluate fitness and parasite resistance (Raberg and Stjernman 2003), and to determine West Nile virus antibody titres in suspected avian hosts (Ebel et al. 2002). Pesticide (carbendazim) exposure of domestic chickens was associated with decreased serum immunoglobulin levels (and therefore decreased immunocompetence) as measured by ELISA (Singhal et al. 2003). Conversely, embryonic

exposure to lead had no effect on immunoglobulin production in chickens (Lee et al. 2002).

1.7.2.2 Assessment of Cell Mediated Immunity

In the effector phase of the cell-mediated immune response, T lymphocytes recruit macrophages to the site of antigen interaction. During this reaction, macrophage activation and inflammation may cause surrounding tissue injury. This type of localized insult to cells and tissue is termed a hypersensitivity response, and the entire reaction is called delayed-type hypersensitivity (DTH) (Abbas 2005). The antigen-specific test used to measure the DTH response is commonly performed on experimental animals to assess the integrated immune response requiring T cells, antigen presenting cells, cytokines, etc. The test animal is initially sensitized with a protein antigen in adjuvant (e.g., bovine serum albumin, dinitrophenol-keyhole limpet hemocyanin (DNP-KLH), mycobacterium, etc.) administered subcutaneously. Days to weeks later, the animal is challenged by subcutaneous or intradermal injection of the same antigen, and the magnitude of the reaction (assessed by determining the degree of skin inflammation) is measured after 24 to 48 hours (Smits and Janz 2005). Local inflammation develops at the site of antigen injection due to leukocyte recruitment and effector T cell accumulation. As a subtle measurement of the complex cellular reactions involved in cell-mediated immunity, the DTH test can be used to evaluate the strength of the CMI response in animals exposed to immunotoxicants (Abbas 2005).

The DTH test has been used in mallards (*Anas platyrhynchos*) exposed to selenomethionine (SeM) in drinking water (Fairbrother and Fowles 1990) and in chickens exposed to lead *in ovo* (Lee et al. 2001, Lee et al. 2002). Birds treated with SeM showed a reduced DTH response compared to control birds. Although the difference was not statistically significant, the test showed a relationship between the size of the reactive area around the injection (amount of inflammation) and SeM dose, leading the authors to conclude that the DTH test was a potentially valuable method for detecting immunotoxic effects on the CMI system in birds (Fairbrother and Fowles 1990). The immunotoxic effects of lead were demonstrated by a reduced DTH response following *in ovo* exposure of chicken embryos on day 12 of incubation (Lee et al. 2001, Lee et al. 2002). Other studies investigating the immunotoxic effects of lead have also validated the use of the DTH test as a useful *in vivo* method to assess cell-mediated modulation of the immune system (Chen et al.

1999, McCabe et al. 1999), and a suitable biomarker for the assessment of xenobiotic-induced immunotoxicity (Bunn et al. 2000).

1.8 Study Species

1.8.1 Domestic Chicken (*Gallus gallus*): Upland Game Bird Model

The domestic chicken (*Gallus gallus*) is the species which has been most commonly used to study the effects of chemicals on birds. Chickens are a preferred avian model because they are readily available at any time of the year, inexpensive, easy to maintain, and well understood with regard to normal physiology. Domesticated wild birds such as bobwhite quail (*Colinus virginianus*) have replaced chickens as models for standardized avian toxicity tests in pesticide and chemical safety assessment, being more closely related to upland game birds. However, chickens are members of the same avian order (galliformes), and have many characteristics in common. Chickens continue to be used to test veterinary drugs in poultry research, and there is much interest in the chicken embryo model to evaluate the hazards of xenobiotic exposure during specific periods of development *in ovo* (Bloom 1980, Hill and Hoffman 1984, Hoffman 1990a, Schafer 1990, DeWitt 2005).

The avian embryo is a useful model for the study of developmental effects of xenobiotic exposure. Unlike viviparous animals, the fertilized avian egg is a contained system in which the embryo develops without interaction with the mother via the placenta (Bloom 1980, Tazawa and Whittow, 2000). This makes it possible to evaluate the toxic effects of a specific dose of chemical to the developing bird through injection or topical application. External application of xenobiotics such as pesticides and petroleum hydrocarbons to avian eggs has been successfully used in numerous studies of environmental contaminants (Hoffman and Albers 1984, Hoffman 1990a, Lusimbo and Leighton 1996, Sewalk et al. 2001, Hoffman et al. 2004). Topical application, via spraying, painting, or dipping, appears to produce a more uniform response than chemical delivery by injection, and it avoids the potential for infection or trauma to the embryo (Hill and Hoffman 1984). The eggs of avian species nesting in fields that receive agrochemical treatment are at significant risk of topical exposure by direct spray or transfer from the contaminated feathers of incubating parents. Therefore, the chick embryo test system is an important component in the evaluation of potential impacts of pesticides on wildlife (Várnagy 1999).

Although toxic effects observed following *in ovo* herbicide exposure are often subtle, data from egg residue analysis have repeatedly confirmed the transfer of externally applied chemicals into the egg (Somers et al. 1974, Castro de Cantarini et al. 1989, Várnagy 1999). The genotoxic and immunotoxic potential of several commonly used herbicides have previously been investigated by *in ovo* exposure in the chicken. Injection of commercial 2,4-D formulation was shown to cause acute toxic effects (increased mortality), sister chromatid exchange induction, and cytokinetic changes in developing chicken embryos (Arias 1994, 2000 & 2003). External 2,4-D application was associated with alterations in hepatic lipid content and enzyme activities, as well as neurotoxic effects (Mori de Moro et al. 1985 & 1986, Evangelista de Duffard et al. 1993).

1.8.2 Domestic Duck (*Anas platyrhynchos*): Waterfowl Model

The domestic mallard duck (*Anas platyrhynchos*) is often used in conjunction with bobwhite quail as an avian model in standardized toxicity tests for safety assessment of new pesticides and other chemicals (Schafer 1990). Mallards have been a primary avian model for the National Pesticides Monitoring Program in the United States since the 1960s, and along with American black ducks (*Anas rubripes*), are used to monitor levels of pesticides in mixed aquatic and terrestrial habitat types (Hill and Hoffman 1984). The domestic mallard is a particularly relevant model for studying the effects of herbicides on wild waterfowl. Several common species of wild ducks and geese are potentially exposed to herbicide spray every spring in the important prairie pothole waterfowl breeding area. Using a closely related domestic species minimizes the uncertainty in extrapolating test results to wild species, while enabling access to a consistent supply of fertile eggs.

As is the case with chickens, routine test methods have also been developed for the study of embryotoxicity and teratogenicity using mallard eggs, including evaluation of effects at different times of incubation. A study by Hoffman and Albers (1984) documented the embryotoxic effects of 42 environmental contaminants applied externally to mallard eggs. Results were reported for various petroleum pollutants, insecticides, and herbicides, including 2,4-D and a commercial mixture containing bromoxynil and MCPA. 2,4-Dichlorophenoxyacetic acid was found to be only slightly toxic in aqueous emulsion, but increasingly toxic when applied in an oil vehicle. The LC_{50} for bromoxynil with MCPA in aqueous emulsion was less than 10 times (~7.5x) the recommended field application rate,

with edema, eye malformations, and stunted growth reported in some survivors (Hoffman and Albers 1984).

1.9 Research Goal and Objectives

1.9.1 Goal

The overall goal of this research project was to assess the effects of *in ovo* commercial herbicide exposure in newly hatched chickens (*Gallus gallus*) and ducks (*Anas platyrhynchos*). To best represent true environmental conditions, fertile eggs were sprayed (either early or late in incubation) with one of two commonly used herbicide formulations at field application rates. In three separate experiments, chicken eggs were sprayed with 2,4-D ester formulation or with Buctril-M® formulation, and duck eggs were sprayed with 2,4-D ester formulation. Genotoxicity and immune function were evaluated in the hatchlings as the main toxicological endpoints to assess potential subtle effects from herbicide exposure, but additional measures of general health and development were also evaluated. Two endpoints were used to assess subtle changes to genetic integrity. The comet assay was used to detect structural damage (strand breaks) in avian lymphocyte DNA, as an index of acute genotoxic effects. Flow cytometry was used to examine potential clastogenic effects of the herbicides, by determining if chromosomal changes resulted in variability in the DNA content of avian erythrocytes. Several endpoints were examined to evaluate potential exposure-induced effects on the immune system. Immunological assessment of chicks and ducklings included differential leukocyte counts, as well as immune organ weights and histopathology. The cell-mediated and humoral immune responses in hatchlings were assessed using the delayed-type hypersensitivity test and measurement of systemic antibody production in response to immunization, respectively. Hematology and hatchling growth rate were also evaluated as indicators of general health.

1.9.2 Objectives

The specific objectives of this research were:

1. To determine if *in ovo* 2,4-D exposure affects genetic integrity of domestic chickens and ducks by:
    a. measuring DNA damage (strand breaks) in isolated peripheral blood lymphocytes using the comet assay;

b. assessing chromosomal damage (as DNA content variability) in peripheral blood erythrocytes using flow cytometry.

2. To determine if *in ovo* 2,4-D exposure causes immunomodulation in domestic chickens and ducks by:
   a. assessing the humoral immune response by measuring systemic antibody production following immunization;
   b. assessing cell-mediated immunity using the delayed-type hypersensitivity test;
   c. examining histopathological changes in primary and secondary immune organs, physiological changes in the relative size of the bursa of Fabricius and spleen, and hematological variables.
3. To determine if *in ovo* Buctril-M® exposure affects genetic integrity of domestic chickens by:
   a. measuring DNA damage (strand breaks) in isolated peripheral blood lymphocytes using the comet assay;
   b. measuring chromosomal damage (as DNA content variability) in peripheral blood erythrocytes using flow cytometry.
4. To determine if *in ovo* Buctril-M® exposure causes immunomodulation in domestic chickens by:
   a. assessing the humoral immune response by measuring systemic antibody production following immunization;
   b. assessing cell-mediated immunity using the delayed-type hypersensitivity test;
   c. examining histopathological changes in primary and secondary immune organs, physiological changes in the relative size of the bursa of Fabricius and spleen, and hematology endpoints.

CHAPTER 2

GENOTOXIC EFFECTS OF *IN OVO* 2,4-D EXPOSURE IN DOMESTIC CHICKENS (*GALLUS GALLUS*) AND DUCKS (*ANAS PLATYRHYNCHOS*)

Abstract

Agricultural practices such as reduced tillage and fall seeding result in increased vegetative ground cover in the early spring compared with conventional approaches. In northern prairie habitat, this sparse cover provides preferred nesting sites for waterfowl and upland game birds. The nesting period for these species often coincides with herbicide treatment of many important cereal crops. Therefore, eggs of ground nesting birds have the potential to be exposed during routine spray applications. A common herbicide formulation used for weed control on the Canadian prairies is 2,4-dichlorophenoxy acetic acid (2,4-D). Previous studies indicate the potential concern for sublethal effects of this herbicide on developing birds, including possible DNA damage. The present study assessed the effects of *in ovo* exposure to a 2,4-D ester herbicide formulation on genetic integrity in newly hatched domestic chickens (*Gallus gallus*) and ducks (*Anas platyrhynchos*) as surrogates for wild galliformes and waterfowl.

Fertile eggs of both species were sprayed with the herbicide at either the normal field application rate, or at 10 times the recommended rate, on days 6 or 15 (chickens) and 6 or 21 (ducks) of incubation, to evaluate risks from herbicide exposure during early or late developmental stages, respectively. Control groups consisted of eggs sprayed with water only. Potential damage to genetic material was evaluated using two genotoxicity assays. The comet assay was used to measure DNA strand breaks in peripheral blood lymphocytes collected from 7-day-old birds, and flow cytometry was used to evaluate DNA content variability in circulating erythrocytes collected from 21-day-old birds. In the comet assay, DNA strand breaks are detected as fragments or uncoiled loops that migrate away from nuclear DNA during electrophoresis to form a measurable “tail”, and damage is quantified using three measurements: comet tail length, percent DNA in the tail, and tail moment (tail

length multiplied by the % DNA in the tail). Flow cytometric analysis estimates the variability in DNA content among a specific population of cells. DNA content differences among individual cells (measured in 10,000 erythrocyte nuclei) are reported as the half peak coefficient of variation (HPCV), a measure which increases following exposure to clastogenic agents and subsequent unequal distribution of chromosomal material during mitosis.

The association between herbicide exposure and genotoxicity endpoints was analyzed using a mixed linear statistical model. Herbicide treatment and time of exposure were accounted for as fixed factors. The results suggest that *in ovo* commercial 2,4-D exposure does not have a significant effect on genetic integrity in domestic chickens, as measured using the comet assay and flow cytometry ($P > 0.05$). *In ovo* 2,4-D treatment also did not significantly affect the variability in DNA content in domestic duck erythrocytes as measured by flow cytometry ($P > 0.05$). There was an association between time of spray exposure (day 6 versus day 21 of incubation) and the amount of DNA strand breaks observed in the comet assay, based on percent DNA in the comet tail ($P = 0.03$). Increased DNA strand breaks were evident in lymphocytes from ducklings sprayed during early incubation. However, since the association between herbicide treatment and strand breaks was not significant, the differences in strand breaks observed in association with time of spraying cannot solely be attributed to 2,4-D treatment. The stress of the manipulations associated with egg handling and spraying should be considered, and duck eggs may be more vulnerable to this physical stressor than chickens because day 6 represents an earlier developmental stage for the ducks. DNA strand breakage, as represented by the comet metrics tail moment and tail length, was not significantly different from the controls ($P > 0.05$) when time of exposure was considered as a factor of effect.

2.1 Introduction

Over the last two decades, significant efforts have been made to reduce negative environmental impacts of agriculture in Canada without impacting crop production. New agricultural practices implemented for conservation purposes include reduced soil tillage and the use of fall planted crops. Reduced tillage and increased planting of winter cereals (so-called “sustainable agricultural” techniques) are strategies employed to preserve soil and water resources, and to maintain wildlife habitat and biodiversity, especially on the prairies.

Fields that receive no-till and/or winter cereal seeding generally provide superior wildlife habitat, especially for ground-nesting birds in the spring. A potential disadvantage of this habitat-friendly practice is the reliance of farmers on herbicide products to control weeds. Using herbicides is usually the only alternative to tilling to discourage weed growth in seeded fields, and herbicide application rates are typically higher in low or no till fields (Gebhardt et al. 1985, Campbell 1999). 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most commonly used herbicide in prairie agriculture (Fowler 2002). Because typical spraying periods for spring weed control overlap with the nesting period of many species of ground-nesting waterfowl and upland game birds, the risk of egg exposure to 2,4-D is significant. The potential long-term effects of low rates of exposure to 2,4-D in wildlife, including avian species, is poorly understood, especially when exposure occurs during embryonic development. Therefore, because conservation practices may increase the risk of embryonic exposure to 2,4-D in critical prairie pothole breeding areas, it is important to investigate potential sublethal effects of this herbicide on developing birds.

Interest in genotoxic effects of exposure to environmental contamination is rapidly growing as part of a desire to better understand subtle and sublethal mechanisms of toxicity in humans and wildlife. Genotoxic agents may produce adverse effects at the cellular level, resulting in structural changes to DNA, including strand breaks, adduct formation, base modifications, etc.. These alterations can be used as biomarkers of exposure, to complement studies of genotoxicological diseases, such as carcinogenesis (Shugart 1999, Shugart et al. 2003) as markers of effect. Indeed, the purpose of a useful biomarker is to be able to reveal whether organisms have been exposed to potentially toxic substances, and to indicate the magnitude of the organism's response to exposure, preferably before any lethal effects occur. Furthermore, studying markers of genotoxic effects may ultimately reveal other population-level effects that result from critical contaminant-induced genetic changes (Kleinjans and Schooten 2002, Shugart et al. 2003).

The toxicity of 2,4-D has been extensively studied and debated. Certain effects of 2,4-D exposure have been well documented, but studies of the action of this herbicide on genetic material (i.e. the potential for 2,4-D to be carcinogenic, mutagenic, and/or genotoxic) are contradictory. Some studies have shown mutagenic and clastogenic effects in mammalian cells after 2,4-D exposure (Venkov et al. 2000, Amer and Aly 2001), while

others have demonstrated no chromosomal effects (Gollapudi et al. 1999, Charles et al. 1999a & 1999b). In avian models, 2,4-D has exhibited toxic action similar to peroxisome proliferators, such as induction of sister chromatid exchange, micronuclei, and clastogenicity (Arias 1994 & 2003). In the present study, the potential genotoxic effects of 2,4-D on developing birds was evaluated using two different assays to assess DNA integrity following *in ovo* herbicide exposure. The comet assay and flow cytometric analysis were used to measure potential DNA strand breaks and clastogenic damage, respectively, in both domestic chickens and ducks.

DNA strand breakage occurs at baseline levels under natural, physiological conditions in all cells. However, exposure to genotoxic agents may cause a significant increase in the frequency and/or severity (i.e. increased unrepairable and potentially inheritable lesions) of DNA damage (Shugart and Theodorakis 1998). The alkaline comet (single cell gel electrophoresis) assay is used to detect various types of strand breaks (single, double, and alkali-labile sites expressed as strand breaks) in DNA, which may be indicative of contaminant exposure (Brendler-Schwaab et al. 2005). In this assay, breaks become visible after cellular suspensions undergo cell lysis and DNA unwinding, followed by electrophoresis which causes uncoiled DNA or DNA fragments to migrate out of the nucleus, forming a measurable "comet tail". After comets are visualized with fluorescent dye, the extent of DNA damage can be quantified by measuring the size and fluorescent intensity of the tail (Tice et al. 2000). Metrics used to evaluate DNA strand breakage in the comet assay are usually based on measuring the amount of DNA in the tail (the damaged DNA) as a proportion of the total nuclear DNA. There is no consensus as to the best metric, but the most commonly used include comet tail length (measured from the leading edge of the head to the tip of the tail, in μm), tail DNA content (percent DNA in the tail), and tail moment (tail length multiplied by the percent DNA in the tail).

Increases in DNA fragmentation as a result of contaminant exposure have been documented in numerous studies investigating genotoxic effects of environmental contaminants in various species, including mammals, amphibians, and avian wildlife (Pandrangi et al. 1995, Nacci et al. 1996, Ralph et al. 1996, Clements et al. 1997, Pastor et al. 2001a & 2001b, Ateeq 2005). Studies using the comet assay have demonstrated the association of increasing genetic damage to increases in the size and stain intensity of the

comet tail. Therefore, assessment of structural damage to DNA based on measurement of strand breakage has been shown to represent a valid biomarker of genotoxic effect.

Structural alterations to DNA that remain unrepaired may also potentiate irreversible chromosomal changes within a cell. During the process of DNA replication and cell division, clastogenic damage may alter the proper (i.e., equal) allocation of chromosomes into daughter cells, resulting in abnormal cells that contain different amounts of DNA. The DNA content of a population of cells can be measured using flow cytometry. The degree of DNA content variability among the population of cells (as measured by either the coefficient of variation, CV, or half-peak coefficient of variation, HPCV) gives an indication of the extent of clastogenic damage. A number of wildlife field studies have used flow cytometry to investigate the impacts of environmental genotoxicants. These studies have demonstrated that either increased CV or HPCV of DNA content is a useful biomarker to detect subtle changes in the genetic integrity of wild species (Bickham et al. 1988, George et al. 1991, Bickham et al. 1992, Bickham et al. 1994, Custer et al. 1994, Lamb et al. 1995, Lowcock et al. 1997, Custer et al. 2000, Matson 2004). Flow cytometry has been used to demonstrate chromosomal damage in birds in association with exposure to petroleum hydrocarbons (Custer et al. 1994), radioactive waste (George et al. 1991), and polycyclic aromatic hydrocarbons (Custer et al. 2000, Matson et al. 2004). However, to the author's knowledge, there are no published reports of the use of this technique to assess chromosomal damage in birds exposed to pesticides.

The purpose of this study was to investigate whether *in ovo* exposure to a commercial 2,4-D herbicide spray formulation at two different times during incubation was associated with DNA damage in domestic chickens (*Gallus gallus*) and ducks (*Anas platyrhynchos*). Potential genotoxic effects of herbicide exposure were assessed using the comet assay to evaluate increased DNA strand breaks in isolated peripheral blood lymphocytes from 7-day-old hatchlings, and flow cytometry to measure chromosomal damage in circulating erythrocytes from 21-day-old hatchlings. The *in ovo* herbicide exposure design was intended to simulate a scenario in which eggs of ground nesting waterfowl or upland game birds are sprayed with herbicide during weed control operations at either early or late incubation timepoints. The results of this study will help to determine the subtle impacts of 2,4-D on different stages of avian embryonic development.

2.2 Materials and Methods

2.2.1 Animal Model

Fertile chicken (*Gallus gallus*) eggs from a White Leghorn/Brown Leghorn cross were incubated at 37.5°C and approximately 80% humidity in circulated air incubators (Hova-Bator®, G.Q.F. Manufacturing Co., Savannah, Georgia, USA) until 1 day post-hatch (about day 23). Automatic egg turners (Hova-Bator®, G.Q.F. Manufacturing Co., Savannah, Georgia, USA) were used for the first 18 days of incubation. On day 19 of incubation, egg turners were removed and eggs were placed on the wire floor of the incubator. Humidity was increased to approximately 90% in accordance with hatching requirements. Chicks were transferred to heated brooders with raised wire floors at one to two day(s) of age, and maintained on *ad libitum* chick starter and fresh water for the duration of the study. Fertile Pekin duck eggs, a domestic mallard (*Anas platyrhynchos*) breed, were raised in a similar fashion with the following modifications. Duck eggs were incubated at > 90% humidity for approximately 28 days until hatch, then transferred to dry incubators until 1 day post-hatch. Automatic egg turners were used for the first 25 days of incubation, and removed on day 26. Ducklings were transferred to pens with solid heated floors bedded with aspen shavings or straw for the remainder of the study.

2.2.2 Herbicide Spray Exposures

Fertile chicken and duck eggs (3 replicate groups each of 120 eggs) were randomly assigned to one of six incubators (20 per incubator), and each incubator was randomly assigned to a specific treatment. Different types of developmental effects may be attributed to genotoxicant insult during specific periods of embryonic development. In order to account for time-specific vulnerability of the embryos, eggs were exposed to the herbicide during either an early (day 6 for chickens and ducks) or late (day 15 for chickens and day 21 for ducks) incubation stage (DeWitt et al. 2005).

Eggs were sprayed with a commercial formulation of 2,4-D ester (Interprovincial Cooperative Limited Agri Products Department, Winnipeg, Manitoba, Canada) at one of two different concentrations: 1) Low dose groups (early and late) were sprayed with 2,4-D at the recommended field application rate for winter wheat (0.56 L ai ha^{-1}) (SAFRR 2005). This was equivalent to 4.24 ml of 2,4-D per litre of herbicide solution. 2) High dose groups (early and late) were sprayed with 42.4 ml 2,4-D per litre, equivalent to 10

times the recommended concentration of herbicide, to simulate a worst-case exposure. Additional groups of eggs (early and late) were sprayed at the same time points with water only to act as negative control groups. The spray treatments were applied using an agricultural field spray simulator (Research Instrument Company, Guelph, Ontario, Canada) (Figure 2-1) at the Agriculture and Agri-Foods Canada Research Centre in Saskatoon, Saskatchewan, to reproduce actual field application conditions as closely as possible. Eggs were masked prior to spraying to ensure that every egg received similar amounts of herbicide (Figure 2-1). The six treatment groups comprising each replicate were: high 2,4-D dose (early and late incubation exposures); low 2,4-D dose (early and late); and negative control (water only, early and late). The three replicates for chicken and duck experiments were spaced about two weeks apart.

2.2.2.1 Quantifying Herbicide Exposure

Herbicide deposition on the surface of the eggs was quantified in a separate study in which surplus eggs of both species were sprayed with fluorescein dye solution (10% (w/w) fluorescein sodium salt in water) at the same application rate as the herbicide treatments. The amount of fluorescein dye deposited on the exposed portion of the eggs was determined by rinsing the eggs to remove the dye, and determining the amount of fluorescein in the rinsate. The rinsate fluorescence was measured at 498 nm using a spectrofluorometer (Shimadzu RF-1501, Shimadzu Corporation, Columbia, MD, USA).

Surplus chicken and duck eggs were collected at 1 and 5 (chickens only) days after spraying on day 6 of incubation, for analysis of 2,4-D residue concentrations in the embryos. After extraction from the shell on the side opposite herbicide deposition, embryos were homogenized (Brinkmann POLYTRON® homogenizer, Brinkmann Instruments, Inc., Westbury, New York, USA) and 2,4-D residues were extracted with acetonitrile (Caledon Laboratories Ltd, Edmonton, Alberta, Canada). Herbicide concentrations in embryo extracts were measured with a high performance liquid chromatograph (LC) (Waters 2695 Alliance System, Milford, Massachusetts, USA) coupled with tandem mass spectrometry (MS/MS) (Waters Micromass® Quattro Ultima™, Milford, Massachusetts, USA). Concentrations of 2,4-D (ng/ml) were determined using a known amount of internal standard (deuterated 2,4-D (d5), Cambridge Isotope Laboratories, Cambridge, Massachusetts, USA), and corrected to

compensate for losses associated with sample processing during the extraction procedure (50%) and for extraction efficiency (50%) of the LC-MS/MS.

2.2.3 Sample Collection

Whole blood is not generally appropriate for use with the comet assay in birds, because > 80% of the red blood cells exhibit the "ghost cell" appearance associated with apoptosis, which is presumably due to degraded and functionally inert DNA/RNA within nucleated, mature erythrocytes (Knopper and McNamee, 2006). Therefore, the comet assay was performed on isolated peripheral blood lymphocytes from 7-day-old chicks and ducklings. Immediately prior to blood collection, a subset of 5 birds per treatment group was randomly selected for the assay, and body weight was measured to the nearest gram using an electronic balance (Mettler PK 4800). Blood samples were collected from the jugular vein with a heparinized syringe into heparinized Microtainer® tubes, and kept on ice, protected from light, until analysis, which occurred within 2 hours of collection. At least 250 µl of whole blood was required from each bird to obtain sufficient numbers of lymphocytes for the comet assay. Flow cytometry was performed on peripheral erythrocytes from all 21-day old chicks and ducklings. Blood was collected from the jugular vein with a heparinized syringe into heparinized Vacutainer® tubes, and a 500 µl aliquot of each sample was mixed in 1.0 ml cryovials (Nalge Nunc International, Rochester, NY, USA) with an equivalent volume of chilled citrate buffer, consisting of 250 mM sucrose, 40 mM trisodium citrate, and 5% v/v DMSO, adjusted to pH 7.6 with 1.0 M citric acid (BDH, Toronto, ON, Canada). Samples were immediately frozen in liquid N_2, and stored at -80°C until flow cytometric DNA analysis could be performed.

Birds were euthanized by cervical dislocation after blood collection on day 21. The use of animals in this research was approved by the University of Saskatchewan Committee on Animal Care and Supply. Birds were housed, handled, and sacrificed in accordance with guidelines established by the Canadian Council on Animal Care.

Unless otherwise noted, all reagents and chemicals were purchased from either EMD Chemicals Inc. (Gibbstown, NJ, USA) or Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada), while laboratory disposables were obtained from VWR International (Mississauga, ON, Canada).

2.2.4 Comet Assay

The comet assay was performed according to procedures outlined by Knopper (2005). This standard method is based on techniques optimized by McNamee et al. (2000) and originally developed by Singh et al. (1988). The agarose solution consisted of 0.75% w/v DNA grade, low melting point (LMP) agarose (Fisher Biotech, Fairlawn, NJ, USA) in phosphate buffered saline (PBS) composed of 58 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 68 mM NaCl, pH 7.4. The lysis buffer (pH 10.0) was prepared with 2.5 M NaCl, 100 mM tetrasodium EDTA, 10 mM Tris base, and 1% w/v *N*-lauryl sarcosine, with the addition of 1% v/v Triton X-100 to required volume 30 min prior to use. The alkaline unwinding (electrophoresis) buffer was prepared fresh on the day of the experiment, with 0.3 M NaOH, 10 mM tetrasodium EDTA, 0.1% w/v 8-hydroxyquinoline, and 2% v/v DMSO, adjusted to pH 13.1 with concentrated NaOH or HCl. Lymphocytes were isolated from whole blood samples using Ficoll-Paque Plus® (Amersham Biosciences Corporation, Piscataway, NJ, USA), following a modification of the procedure recommended by the manufacturer. Three 15 ml Falcon® conical centrifuge tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were numbered and labeled for each blood sample, with 250 µl PBS added to tube #1, 3.0 ml Ficoll-Paque Plus® added to tube #2, and 1.0 ml PBS added to tube #3. Tubes were stored overnight at 4°C. All subsequent steps were performed under reduced light conditions within one hour after blood collection. Whole blood (250 µl) was added to tube #1 to make a 1:1 suspension of blood in PBS. Using a Pasteur pipette, the contents of tube #1 were mixed and carefully layered on top of the Ficoll-Paque Plus® gradient in tube #2. To keep the "layers" separate, the tube and pipette were held at a 45° angle, and the pipette tip was kept about 5 mm above the Ficoll-Paque Plus® as the blood was expelled. All #2 tubes were centrifuged for 30 min at 2000 rpm (Beckman J-6B, Beckman-Coulter, Mississauga, ON, Canada). After centrifugation, the white blood cell (WBC) layer (buffy coat) was withdrawn with a pipette, and was added to tube #3. After mixing, the tubes were centrifuged for 10 min at 2000 rpm, and the supernatant was poured off. The WBC pellet was resuspended in a known volume (usually 500 µl) of PBS, and placed on ice. Within one hour of the assay, lymphocyte viability was assessed using the Trypan blue exclusion test. Only samples showing greater than 90% lymphocyte viability were used in the assay. A 50 µl aliquot of the WBC suspension was added to 50 µl of Trypan blue working solution, consisting of a

1/40 dilution of Trypan blue stock in a 1% v/v acetic acid solution in saline. The cell suspension was loaded onto a hemocytometer and live and dead WBCs counted using a light microscope at 40X. Cell viability was calculated to maintain consistency among samples (as compared to the control sample), and to assess cytotoxicity in the cell suspension to determine the cause (genotoxic or otherwise) of cell damage (Tice et al. 2000, Knopper 2005). The following calculations demonstrate the determination of cell viability (and cell concentration):

1) WBC concentration in PBS =

Average of total WBC count (live + dead) * dilution factor * conversion factor (10,000)

E.g. 25 WBC * 2 * 10,000 = 500,000 or 5 x 10^5 cells/ml PBS

2) WBC concentration in whole blood = WBC concentration in PBS * 4 (250 µl sample)

E.g. 5 x 10^5 cells/ml PBS * 4 = 2 x 10^6 cells/ml whole blood

3) WBC viability =

(Average live cells (of four corner count) / Average total cells (live + dead)) * 100

E.g. (25 live cell average / 27 total cell average) * 100 = 92.6% WBC viability

All subsequent steps of the comet assay were performed in subdued light. A 30 µl aliquot of the purified WBC suspension was added to 270 µl of liquefied 0.75% agarose and gently mixed. Aliquots (120 µl each) of the cell/agarose mixture were then cast (in duplicate) into individual wells of a two-well Lab-Tek® chamber (Nalge Nunc International, Rochester, NY, USA) affixed to GelBond® film (FMC Bioproducts, Rockport, ME, USA). Each piece of film supported three chambers (three different samples, in duplicate). Internal control samples were also cast simultaneously into four wells, to represent negative and positive controls, designed to assess assay performance and comet formation, respectively. Once the agarose solidified (approximately two min), the Lab-Tek® chambers were carefully removed, leaving the agarose-embedded cells attached to the films. Positive control films were exposed for five min to ice-cold, freshly prepared 1 mM H_2O_2 in PBS. Remaining films were each immersed in 50 ml ice-cold lysis buffer, and maintained at 4°C in the dark for 60 min. After lysis, films were gently rinsed with distilled water (ddH_2O) and placed into 50 ml fresh electrophoresis buffer for 30 min at room temperature to allow the DNA to unwind. Electrophoresis was subsequently performed in chilled Hoefer HE33 gel electrophoresis units (Hoefer Scientific Instruments, San Francisco, CA, USA) containing

220 ml electrophoresis buffer. Electrophoresis gel units were powered by a Thermo EC570-90 power unit (Thermo Electron Corporation, Waltham, MA, USA), and films were run for 20 min at 19 V (~1.5 V/cm constant voltage, >300 mA). After films were electrophoresed, they were placed in 50 ml 1 M ammonium acetate neutralization solution for 30 min, then transferred to 95% ethanol for two hours to dehydrate before air-drying overnight. Dry films were labeled and stored in envelopes until imaging analysis.

Image analysis was performed on one set of samples and all control gels. GelBond® films were cut into three strips, each containing one sample. Individual films were stained for 10-15 minutes in a 1/10,000 dilution of stock SYBR Gold® (Molecular Probes, Eugene, OR, USA) in ddH_2O. Films were double rinsed in water, placed onto a glass microscope slide (gel side up), covered with a cover slip (22 x 50 mm), and gently pressed with a cloth to remove excess water and form a seal. Stained slides were examined with a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss Microscopy, Jena, Germany), and comet images were captured with a QImaging Retiga™ 1300 digital CCD monochrome camera (QImaging, Vancouver, BC, Canada). A minimum of 50 cells per slide were scored for DNA migration, and comets were analyzed using Komet version 5.5 comet assay software (Kinetic Imaging, Nottingham, UK) at 430x magnification. The degree of damage was quantified using three different metrics: comet tail length (measured from the leading edge of the head to the tip of the tail, in μm), tail DNA content (% DNA in the tail), and tail moment (tail length multiplied by the % DNA in the tail). Outlier values greater than four standard deviations from the sample mean of the 50 cells were identified and removed. All sample gels were scored without knowledge of the treatment group.

2.2.5 Flow Cytometric DNA Analysis

Flow cytometric DNA analysis was performed on erythrocytes from whole blood samples collected from 21-day-old chicks and ducklings, to determine cell to cell variability in DNA content. The methods used for DNA content analysis followed those previously described by Vindeløv and Christiansen (1994). Unless noted, all solutions (pH 7.6) were prepared up to a week prior to the start of the experiment, and stored at -20°C until needed. A stock solution containing 3.4 mM trisodium citrate, 0.1% v/v IGEPAL CA-630, 1.5 mM spermine tetrachloride, and 0.5 mM Tris base, was used to prepare the remaining solutions, and was kept at 4°C. Solution A consisted of trypsin (30 mg/L stock) and Solution B

contained trypsin inhibitor (500 mg/L stock) and ribonuclease A (100 mg/L). The stain solution contained 3.3 mM spermine tetrahydrochloride and propidium iodide (416 mg/L stock), and was stored in the dark at -20°C.

Samples were sorted prior to processing in order to ensure that each batch of samples analysed on a given day contained a representative from each treatment. Samples from each treatment were randomly chosen to avoid experimental bias. Frozen samples of whole blood were thawed rapidly at room temperature, and prepared for flow cytometric analysis as follows. A clean nuclear suspension was obtained by homogenizing 2 µl of the blood mixture with 50 µl of citrate buffer and 450 µl of Solution A in a microcentrifuge tube, and allowing the samples to sit for 10 min at room temperature. A 375 µl aliquot of Solution B was added to each sample in the microcentrifuge tubes, followed by another 10 min incubation at room temperature. The RNase A component of Solution B degrades double-stranded RNA, leaving only DNA to take up the fluorescent dye during the final step. After 10 min, the nuclear suspension was pipetted through a 37 µm mesh nylon filter cloth (Cole Parmer, Vernon Hills, IL, USA) into a 12x75 mm Falcon™ tube to remove as much cellular debris as possible. Finally, 375 µl of propidium iodide (PI) solution was added to each Falcon™ tube, and incubated for at least 15 min on ice. Samples were analysed on the flow cytometer within two hours of staining.

Nuclear fluorescence was measured on a Coulter Epics Elite® ESP flow cytometer (Beckman Coulter Canada Inc., Mississauga, ON, Canada). Instrument alignment and focus were set with fluorospheres (Flow-Check™, Beckman Coulter) each day, prior to sample analysis. Cells were analyzed at a rate averaging 200-300 cells per second to ensure a thin stream of cells intersecting the laser in a single path. The PI stain was excited using the 488 nm line of an argon ion laser. Fluorescence emission values were measured and plotted as histograms using Expo32® acquisition and analysis software (v.1.2, Beckman Coulter) to estimate the mean and standard deviation of the DNA content in each sample. Ten thousand nuclei in the G1 phase were measured (PMT3 linear vs. PMT3 peak as parameters) from each sample, and, using the histograms generated, the full peak CV (standard deviation/mean x 100, expressed as a percent) and half peak CV (HPCV) were calculated. CV and HPCV describe the width of the histogram peak (DNA content), and therefore represent the

variability in cell DNA content. A wider peak results in an increased CV or HPCV, indicative of greater chromosomal damage.

2.2.6 Statistical Analysis

Descriptive statistics for all outcomes were compiled using SPSS (v.14.0, SPSS Inc., Chicago, IL, USA) and SYSTAT (v.11.0, SPSS Inc., Chicago, IL, USA). Summaries are reported for the subset of animals used for the comet assay (N=79 for chickens, N= 62 for ducks) and for the entire set of animals assessed by flow cytometry (N=199 for chickens, N=84 for ducks). The normal distribution of all assay variables was assessed using the Shapiro-Wilk test for normality. All comet parameters were $\log_{10}$ transformed to attain normality if the p-value given by this test was low (i.e. < 0.5). Correlation (Pearson's *r*) among comet measurements ($\log_{10}$ mean comet tail DNA, tail moment, and tail length) was also determined.

The association between exposure and genotoxicity was analyzed using a mixed linear model (PROC MIXED in SAS v.8.2 for Windows, SAS Institute, Cary, NC, USA). Incubator and experimental group were included as random effects to account for clustering of the observations as a result of separate incubator designations and replicated experiments, respectively. First, time of herbicide exposure (early or late incubation stage) and then herbicide treatment level (high, low concentration and water control) were assessed as fixed effect factors in a model including the random effects. Where time of exposure and herbicide treatment were both potentially important factors ($P < 0.25$), both were included in a model to assess confounding effects. Variables were retained in the final model if they were significant ($P < 0.05$), or acted as important confounders (i.e., adjustment for the variable changed the other coefficient by more than 10%). If both time of exposure and herbicide treatment were significant ($P < 0.05$), the model was then tested for interaction.

2.3 Results

Herbicide deposition on the surface of the eggs was quantified in a separate study in which fluorescein dye was sprayed onto masked eggs, eggs were rinsed, and the amount of fluorescein in the rinsate was measured using a spectrofluorometer. Mean doses of the 2,4-D active ingredient deposited onto masked eggs were calculated for low and high dose exposure groups, and reported in Table 2-1.

2,4-D was detected in chicken and duck embryos collected 1 and 5 (chickens only) days after early (day 6 of incubation) *in ovo* spray exposure. Herbicide residue concentrations in embryos from both treatment levels were determined using LC-MS/MS analysis of egg extracts (N = 3). In chickens, eggs from both high and low dose herbicide treatment groups contained measurable 2,4-D concentrations at 1 (embryonic day 7, E7) and 5 (E11) days after spraying. Embryo 2,4-D concentrations from eggs treated with the low dose concentration increased from a mean of 0.6 ng/g at stage E7 to 2.2 ng/g in eggs collected on E11. A similar trend was observed in chicken eggs treated with the high concentration of 2,4-D, with embryo residue concentrations increasing from 27.4 ng/g at E7 to 374.5 at E11. As expected, higher 2,4-D concentrations were observed in eggs treated at the 10X rate than in those treated with the recommended field application rate (1X). Duck eggs treated with 2,4-D at the 1X application rate contained a mean herbicide concentration of 2.46 ng/g, while eggs sprayed with 10X 2,4-D had 14.1 ng/g.

Figure 2-2 shows two images of alkaline comets from chicken lymphocytes, isolated from whole blood. The viability of purified WBCs used for the comet assay consistently exceeded 90%. Comets from negative (Figure 2-2(a)) and positive (Figure 2-2(b)) assay controls are easily identifiable.

Descriptive statistics for the comet assay outcome variables are summarized in Table 2-2. For all comet metrics, simple comparisons of the assay outcomes from herbicide treated birds to those of the control group revealed only slight differences. Similarities among the variables were reinforced with correlation analysis using the Pearson's coefficient *r*. There was strong correlation among all comet measurements (Table 2-3), with all correlation values showing significance at the 0.01 level.

Table 2-4 summarizes the univariate comparisons between the fixed factors herbicide treatment and time of exposure and all comet variables, in chicken lymphocytes. For the tail DNA content (% DNA in the tail), both treatment ($P = 0.24$) and time ($P = 0.06$) were considered potentially important factors ($P < 0.25$), and were included in the final model. These factors were not important confounders ($P = 0.27$), and neither was considered to contribute significant effects on tail DNA in the final model. For comet tail moment and length, treatment and time were not considered to be important factors in the initial model (*P*

> 0.25). Figures 2-3 and 2-4 show that these factors did not have significant effects on comet tail DNA.

Descriptive statistics for the flow cytometry variable half-peak coefficient of variation (HPCV) are provided in Table 2-5 for all exposure groups in the 2,4-D chicken experiment. Upon analysis with the univariate comparison model, herbicide treatment ($P = 0.99$) and time of exposure ($P = 0.56$) were not considered to be important factors (Table 2-6). Figures 2-5 and 2-6 show the lack of significant relationship between each fixed effect factor and HPCV.

A summary of the comet variables for the 2,4-D duck experiments are provided in Table 2-7. Comet outcomes were strongly correlated, with most of the values showing significance at the 0.01 level (Table 2-8). Both tables indicate that, except for comet tail DNA content, there were few differences among treatment groups. General linear model results are summarized in Table 2-9. For comet tail DNA content, only time of spray exposure was considered an important effect factor ($P = 0.0269$). For comet tail moment, only exposure time approached significance ($P = 0.0639$), and neither herbicide treatment nor time of exposure were significantly associated with comet tail length. Both factors had no effect on comet tail length. Figures 2-7 and 2-8 show that herbicide treatment had no effect on comet tail DNA, but that exposure time was a significant factor, respectively.

Descriptive statistics are summarized in Table 2-10 for the HPCV of DNA content in duck erythrocytes. Univariate comparisons among all exposure groups showed that both herbicide treatment ($P = 0.44$) and time of exposure were insignificant ($P = 0.44$) (Table 2-11). This conclusion is graphically represented in Figures 2-9 and 2-10, respectively.

Figure 2-1. Application of commercial 2,4-D formulation onto masked eggs using an agricultural field spray simulator.

Table 2-1. 2,4-D application rates, spray solution concentrations, and actual doses of active ingredient deposited on eggs in low (1X) and high (10X) exposure groups, as determined by a fluorescein dye retention study (N = 10).

Herbicide Exposure	Application Rate[1] (L ai/ha)	Concentration[2] (ml/L)	Mean Deposited Dose (µg ai/egg) ± SD	
			Chickens	Ducks
1 X 2,4-D (Low Dose)	0.56	4.24	81.7 ± 4.4	87.0 ± 5.8
10 X 2,4-D (High Dose)	0.56	42.40	789.9 ± 53.5	896.3 ± 56.0

[1] Maximum safe application rate of herbicide on wheat crops (litres of active ingredient/hectare) (SAFRR 2005).

[2] Concentration of formulated 2,4-D product (ml) in water spray solution (L).

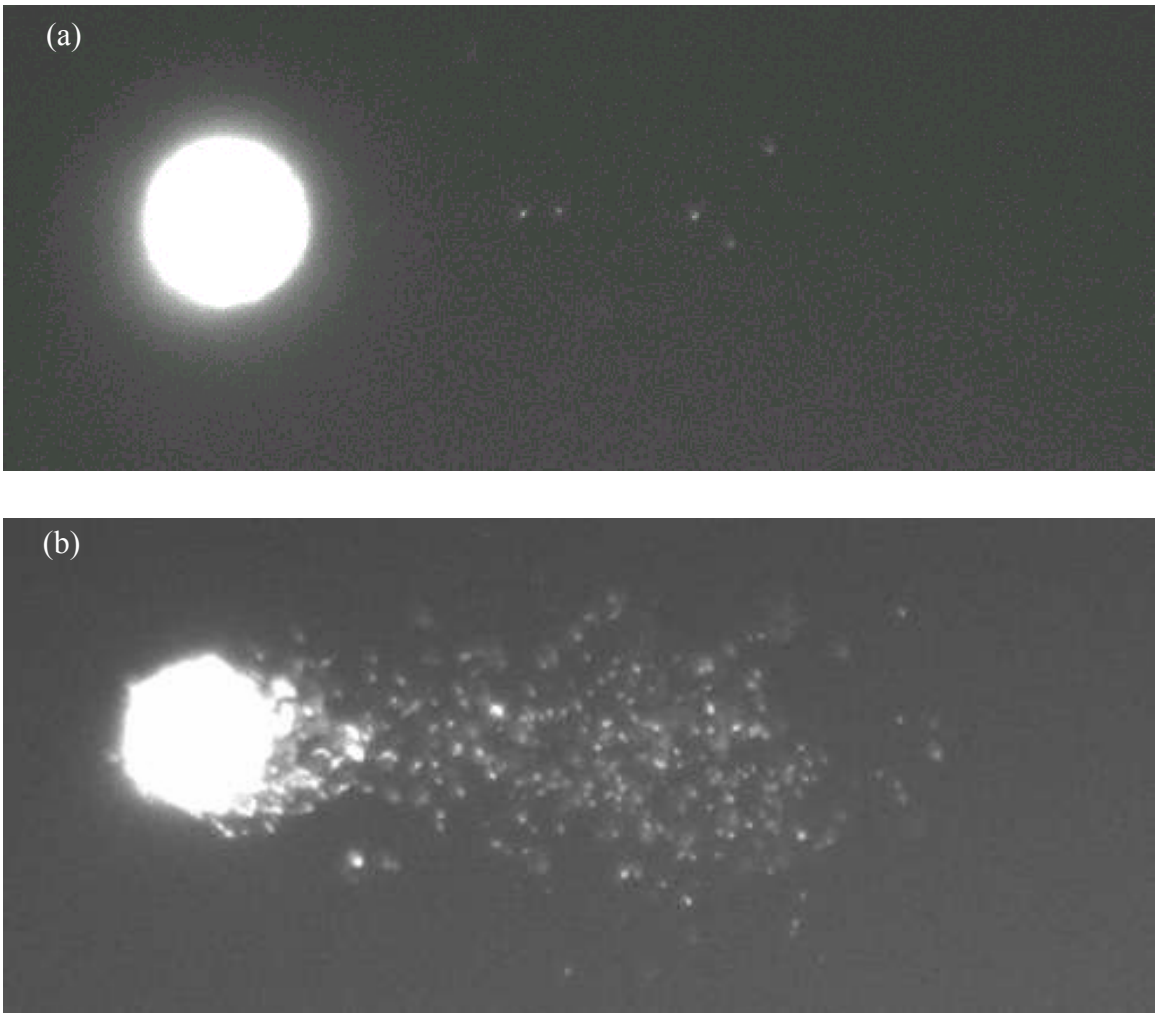

Figure 2-2. Images of alkaline comets from chicken white blood cells with (a) undamaged and (b) damaged DNA (total magnification 430X). Nuclei were stained with SYBR Gold® stain.

Table 2-2. Descriptive summaries of comet assay outcomes (log_{10} transformed to attain normality) from isolated lymphocyte DNA of 7-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray. Summaries are provided for comet tail DNA (a), comet tail moment (b), and comet tail length (c).

(a) **Log_{10} Tail DNA**

							Percentiles	
Treatment	**Exposure Time**	**N**	**Mean**	**SD**	**SE**	**Median**	**25th**	**75th**
Water (Negative Control)	Early[1]	13	1.108	0.196	0.054	1.091	1.000	1.239
	Late[2]	14	1.129	0.187	0.050	1.174	0.980	1.260
1 X 2,4-D (Low Dose)	Early[1]	15	0.971	0.251	0.065	1.076	0.870	1.175
	Late[2]	10	1.133	0.133	0.042	1.125	1.036	1.254
10 X 2,4-D (High Dose)	Early[1]	13	1.037	0.205	0.057	1.055	0.925	1.227
	Late[2]	14	1.089	0.221	0.059	1.054	0.913	1.258

(b) **Log_{10} Tail Moment**

							Percentiles	
Treatment	**Exposure Time**	**N**	**Mean**	**SD**	**SE**	**Median**	**25th**	**75th**
Water (Negative Control)	Early[1]	13	0.394	0.373	0.103	0.416	0.268	0.623
	Late[2]	14	0.410	0.312	0.083	0.491	0.255	0.614
1 X 2,4-D (Low Dose)	Early[1]	15	0.195	0.438	0.113	0.278	0.140	0.542
	Late[2]	10	0.387	0.246	0.078	0.389	0.196	0.585
10 X 2,4-D (High Dose)	Early[1]	13	0.316	0.390	0.108	0.303	0.154	0.589
	Late[2]	14	0.369	0.389	0.104	0.367	0.169	0.573

(c) **Log_{10} Tail Length**

							Percentiles	
Treatment	**Exposure Time**	**N**	**Mean**	**SD**	**SE**	**Median**	**25th**	**75th**
Water (Negative Control)	Early[1]	13	1.524	0.206	0.057	1.570	1.411	1.635
	Late[2]	14	1.494	0.170	0.045	1.523	1.414	1.644
1 X 2,4-D (Low Dose)	Early[1]	15	1.376	0.308	0.080	1.502	1.182	1.584
	Late[2]	10	1.484	0.142	0.045	1.483	1.411	1.545
10 X 2,4-D (High Dose)	Early[1]	13	1.446	0.293	0.081	1.469	1.363	1.582
	Late[2]	14	1.514	0.178	0.047	1.526	1.341	1.634

[1] Day 6 and [2] Day 15 of incubation

Table 2-3. Correlation (Pearson's coefficient, *r*) among chicken lymphocyte comet assay outcomes (log_{10} transformed to attain normality) among all treatment groups; water (control), 1X 2,4-D (low dose), and 10X 2,4-D (high dose), at both early (incubation day 6) and late (incubation day 15) exposure times. Correlation tables are given for the following treatment groups: Control Early (a), Control Late (b), Low Dose Early (c), Low Dose Late (d), High Dose Early (e), and High Dose Late (f).

(a) **Control Early**; N = 13	**Comet Tail DNA**	**Comet Tail Moment**	**Comet Tail Length**
Comet Tail DNA	1.000	0.931*	0.933*
Comet Tail Moment	0.931*	1.000	0.964*
Comet Tail Length	0.933*	0.964*	1.000
(b) **Control Late**; N = 14			
Comet Tail DNA	1.000	0.876*	0.902*
Comet Tail Moment	0.876*	1.000	0.958*
Comet Tail Length	0.902*	0.958*	1.000
(c) **Low Dose Early**; N = 15			
Comet Tail DNA	1.000	0.962*	0.934*
Comet Tail Moment	0.962*	1.000	0.962*
Comet Tail Length	0.934*	0.962*	1.000
(d) **Low Dose Late**; N = 10			
Comet Tail DNA	1.000	0.862*	0.592
Comet Tail Moment	0.862*	1.000	0.821*
Comet Tail Length	0.592	0.821*	1.000
(e) **High Dose Early**; N = 13			
Comet Tail DNA	1.000	0.957*	0.908*
Comet Tail Moment	0.957*	1.000	0.934*
Comet Tail Length	0.908*	0.934*	1.000
(f) **High Dose Late**; N = 14			
Comet Tail DNA	1.000	0.929*	0.854*
Comet Tail Moment	0.929*	1.000	0.949*
Comet Tail Length	0.854*	0.949*	1.000

* Correlation value is significant at the 0.01 level (2-tailed)

Table 2-4. Summary of univariate comparisons between the fixed effect factors herbicide treatment and time of exposure, and comet tail DNA (a), comet tail moment (b), and comet tail length (c) in isolated lymphocyte DNA of 7-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

(a) **Comet Tail DNA**	**Regression coefficient (β)**	**95% Confidence Intervals for β**		***P* value**
		Lower	**Upper**	
Herbicide Treatment				
- High Dose[1]	-0.064	-0.164	0.036	0.20
- Low Dose[2]	-0.082	-0.184	0.019	0.11
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.076	-0.156	0.005	0.06
- Late[5]	Reference	-	-	-
(b) **Comet Tail Moment**				
Herbicide Treatment				
- High Dose[1]	-0.076	-0.245	0.093	0.37
- Low[2]	-0.130	-0.302	0.043	0.14
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.083	-0.222	0.057	0.24
- Late[5]	Reference	-	-	-
(c) **Comet Tail Length**				
Herbicide Treatment				
- High Dose[1]	-0.035	-0.147	0.078	0.54
- Low Dose[2]	-0.088	-0.202	0.027	0.13
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.051	-0.145	0.042	0.28
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

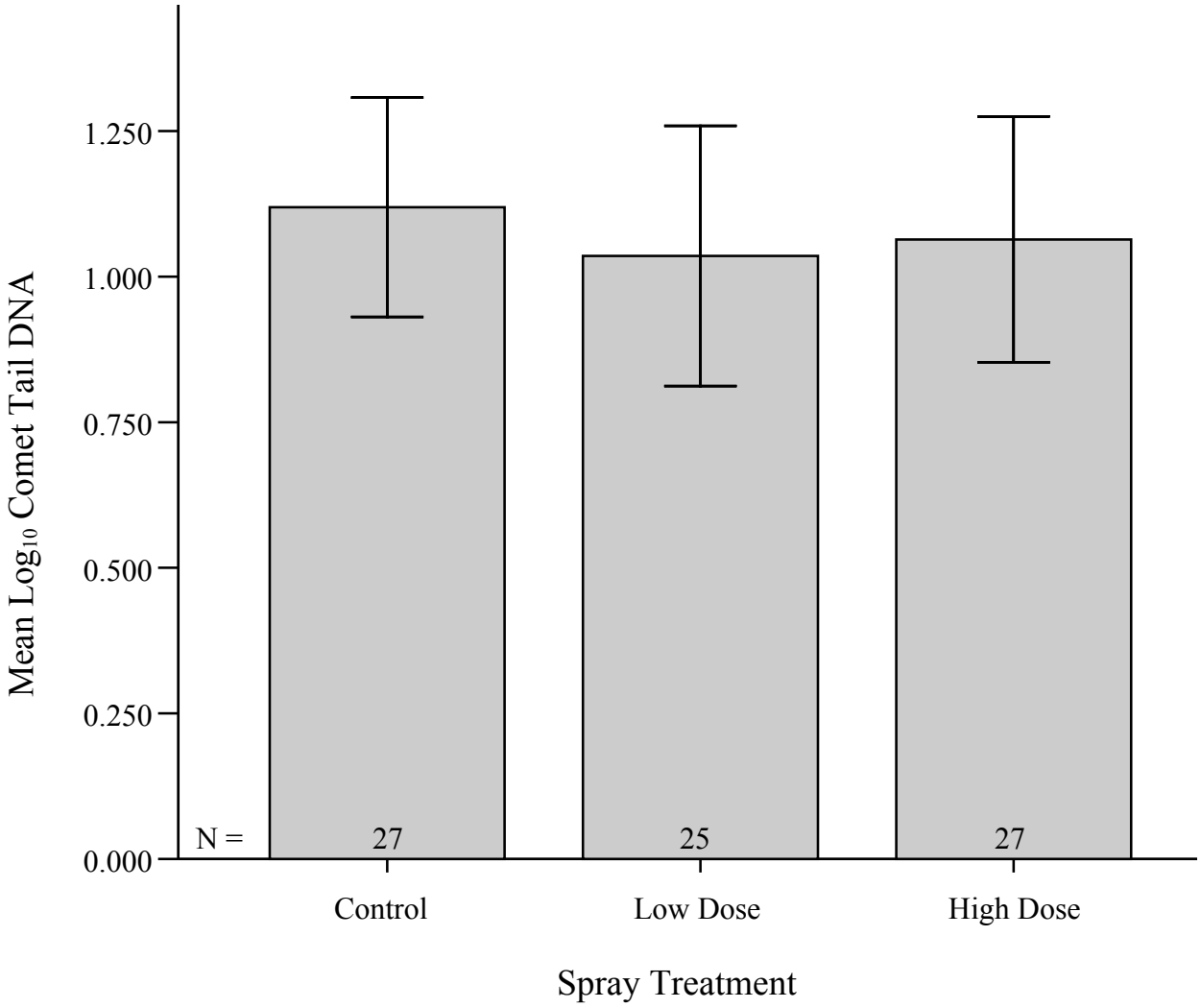

Figure 2-3. The simple association between comet tail DNA in domestic chicken lymphocytes and *in ovo* 2,4-D herbicide exposure. The bars represent mean $\log_{10}$ comet tail DNA for the following groups; control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD.

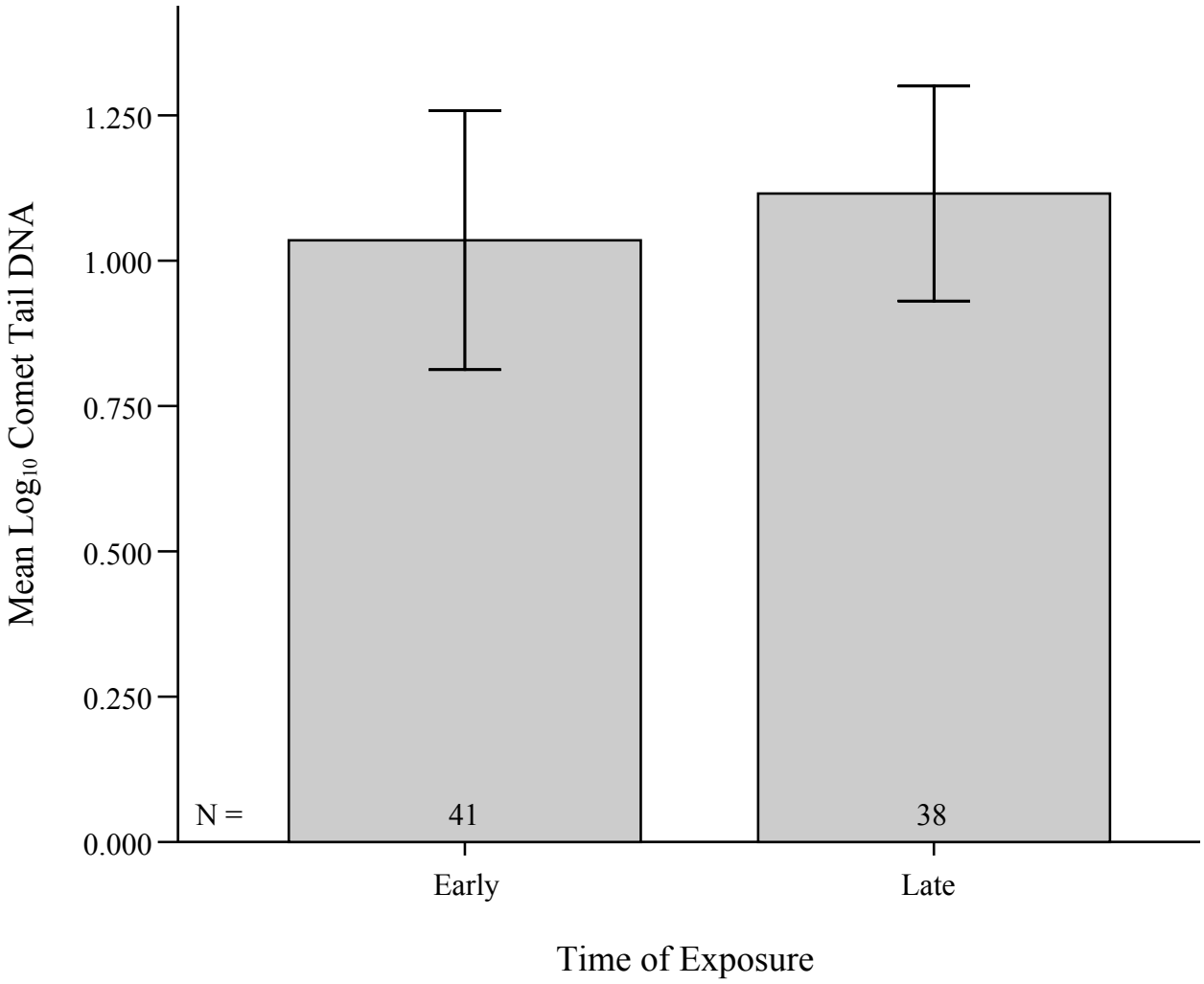

Figure 2-4. The simple association between comet tail DNA in domestic chicken lymphocytes and the time of *in ovo* 2,4-D herbicide spray application. The bars represent mean $\log_{10}$ comet tail DNA for early (day 6 of incubation) and late (day 15 of incubation) exposure groups. Error bars represent ± SD.

Table 2-5. Descriptive summary of flow cytometry outcome, half-peak coefficient of variation (HPCV) in DNA content of circulating erythrocytes, from 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	33	4.42	0.47	0.08	4.40	4.05	4.80
	Late[2]	33	4.50	0.41	0.07	4.50	4.25	4.85
1 X 2,4-D (Low Dose)	Early[1]	31	4.45	0.50	0.09	4.60	3.90	4.80
	Late[2]	34	4.46	0.36	0.06	4.50	4.28	4.80
10 X 2,4-D (High Dose)	Early[1]	34	4.46	0.40	0.07	4.50	4.18	4.73
	Late[2]	34	4.44	0.43	0.07	4.40	4.10	4.80

[1] Day 6 and [2] Day 15 of incubation

Table 2-6. Summary of univariate comparisons between the fixed effect factors exposure time and herbicide treatment, and half-peak coefficient of variation (HPCV) in DNA content of erythrocytes from 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	-0.01	-0.12	0.10	0.92
- Low Dose[2]	-0.01	-0.12	0.10	0.87
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.03	-0.12	0.06	0.56
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

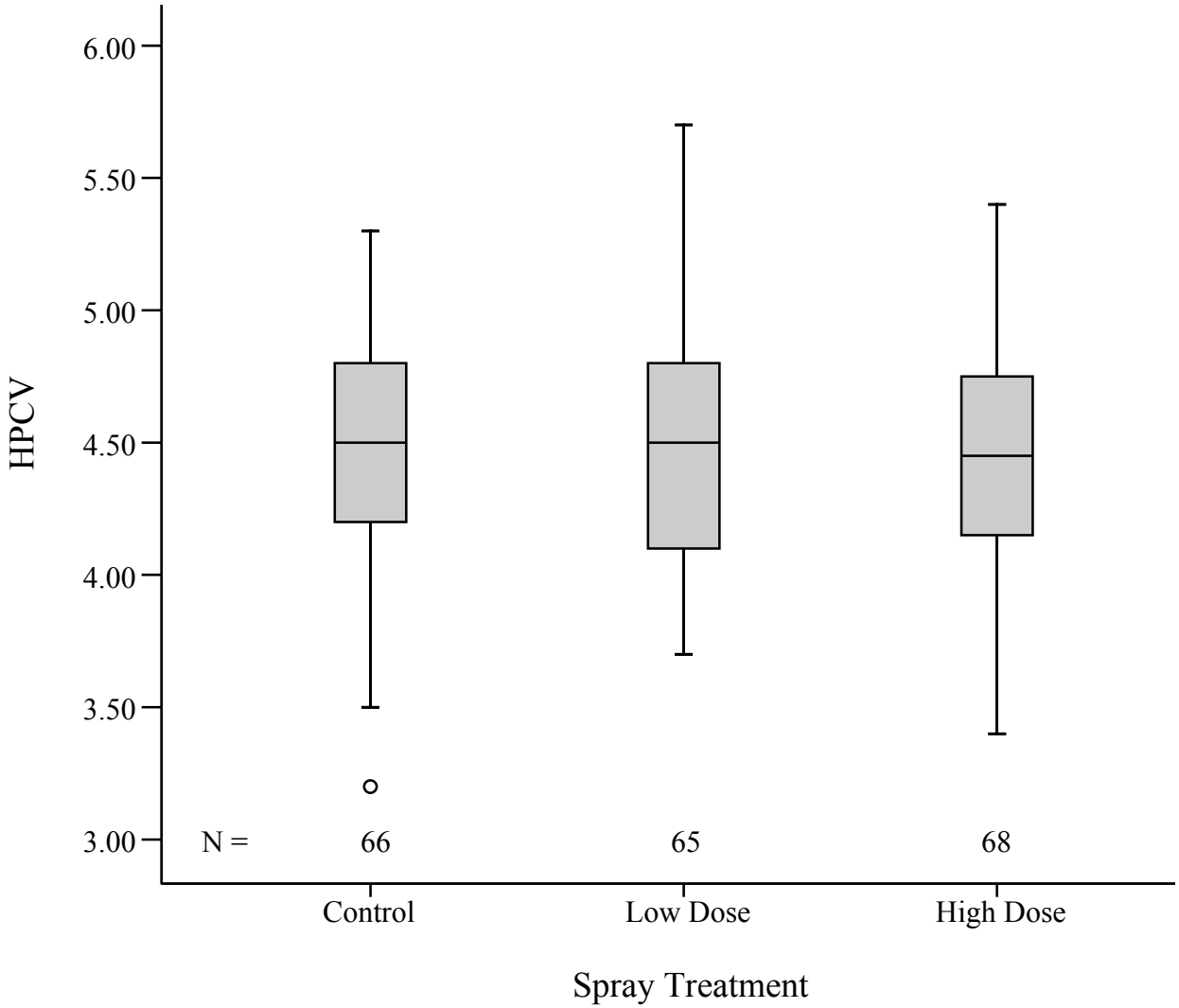

Figure 2-5. The simple association between flow cytometry outcome, half-peak coefficient of variation (HPCV) in DNA content of chicken erythrocytes and *in ovo* 2,4-D herbicide exposure. Boxplots represent the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). The centre line represents the median, the box the interquartile range, the whiskers extend from the highest to lowest values, excluding the outlier, which is represented by ○.

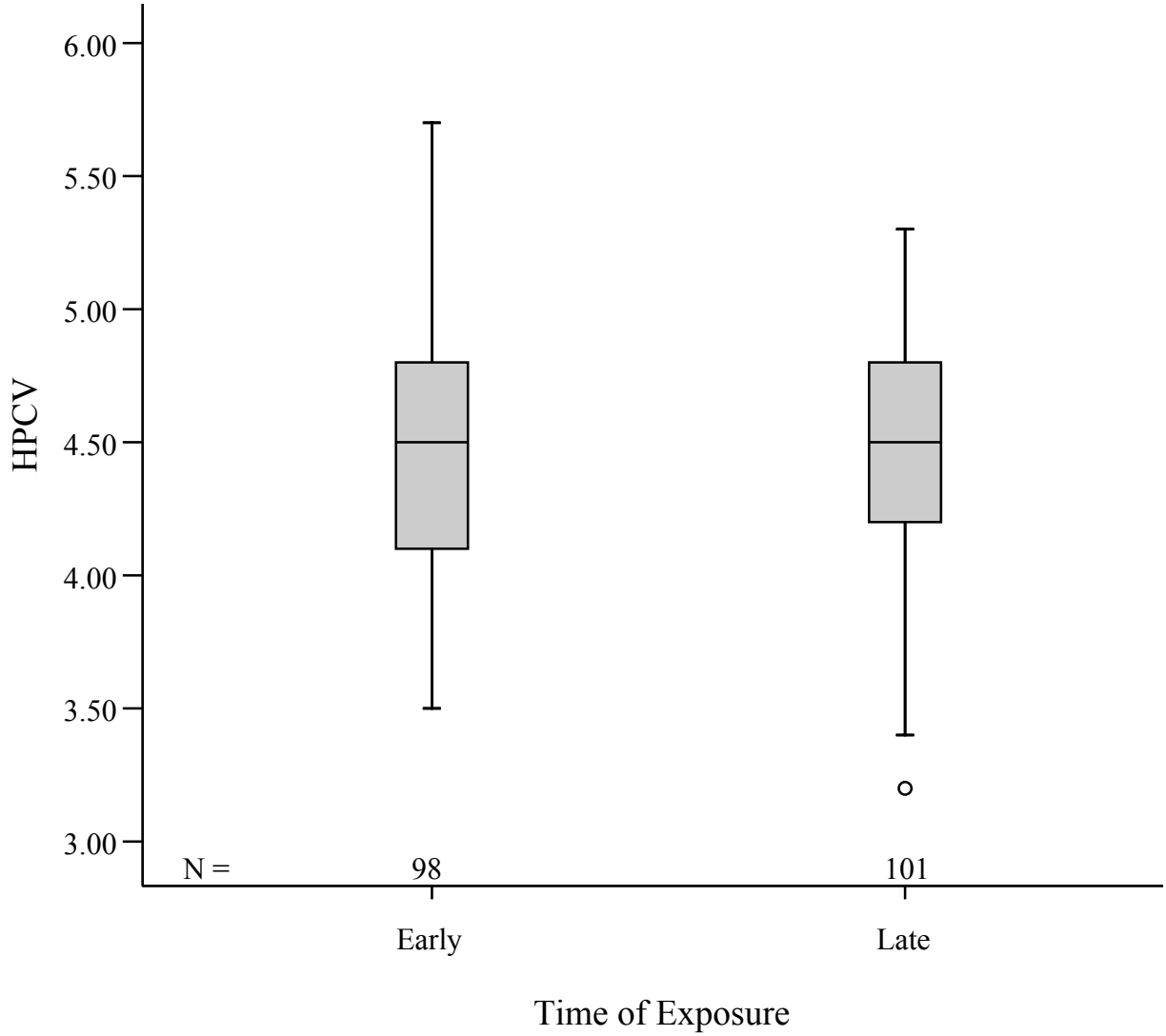

Figure 2-6. The simple association between flow cytometry outcome, half-peak coefficient of variation (HPCV) in DNA content of chicken erythrocytes and the time of *in ovo* 2,4-D herbicide spray application. Boxplots represent the two times of herbicide exposure, early (day 6 of incubation) and late (day 15 of incubation). The centre line represents the median, the box the interquartile range, the whiskers extend from the highest to lowest values, excluding the outlier shown as ○.

Table 2-7. Descriptive summaries of comet assay outcomes (log_{10} transformed to attain normality) from isolated lymphocyte DNA of 7-day-old ducklings exposed *in ovo* to 2,4-D formulation spray. Summaries are provided for comet tail DNA (a), comet tail moment (b), and comet tail length (c).

(a) **Log_{10} Tail DNA**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	14	1.135	0.215	0.057	1.134	0.986	1.264
	Late[2]	14	1.108	0.141	0.038	1.083	1.031	1.229
1 X 2,4-D (Low Dose)	Early[1]	6	1.246	0.136	0.055	1.221	1.167	1.327
	Late[2]	7	1.024	0.215	0.081	1.095	0.807	1.192
10 X 2,4-D (High Dose)	Early[1]	8	1.241	0.191	0.067	1.273	1.065	1.329
	Late[2]	13	1.056	0.158	0.044	1.113	0.877	1.187

(b) **Log_{10} Tail Moment**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	14	0.247	0.346	0.093	0.237	-0.020	0.467
	Late[2]	14	0.194	0.219	0.058	0.146	0.040	0.357
1 X 2,4-D (Low Dose)	Early[1]	6	0.416	0.231	0.094	0.426	0.244	0.536
	Late[2]	7	0.102	0.381	0.144	0.205	-0.349	0.367
10 X 2,4-D (High Dose)	Early[1]	8	0.378	0.218	0.077	0.422	0.205	0.584
	Late[2]	13	0.133	0.234	0.065	0.135	-0.101	0.332

(c) **Log_{10} Tail Length**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	14	1.392	0.186	0.050	1.376	1.235	1.526
	Late[2]	14	1.316	0.162	0.043	1.335	1.202	1.439
1 X 2,4-D (Low Dose)	Early[1]	6	1.456	0.113	0.046	1.453	1.379	1.532
	Late[2]	7	1.274	0.292	0.110	1.352	1.085	1.411
10 X 2,4-D (High Dose)	Early[1]	8	1.457	0.107	0.038	1.451	1.399	1.551
	Late[2]	13	1.298	0.152	0.043	1.343	1.155	1.380

[1] Day 6 and [2] Day 21 of incubation

Table 2-8. Correlation (Pearson's coefficient, *r*) among duckling lymphocyte comet assay outcomes (log_{10} transformed to attain normality) among all treatment groups: water (control), 1X 2,4-D (low dose), and 10X 2,4-D (high dose), at early (incubation day 6) and late (incubation day 15) exposure times. Correlation tables are given for the following treatment groups: Control Early (a), Control Late (b), Low Dose Early (c), Low Dose Late (d), High Dose Early (e), and High Dose Late (f).

(a) **Control Early**; N = 14	**Comet Tail DNA**	**Comet Tail Moment**	**Comet Tail Length**
Comet Tail DNA	1.000	0.988**	0.941**
Comet Tail Moment	0.988**	1.000	0.945**
Comet Tail Length	0.941**	0.945**	1.000
(b) **Control Late**; N = 14			
Comet Tail DNA	1.000	0.954**	0.811**
Comet Tail Moment	0.954**	1.000	0.928**
Comet Tail Length	0.811**	0.928**	1.000
(c) **Low Dose Early**; N = 6			
Comet Tail DNA	1.000	0.965**	0.925**
Comet Tail Moment	0.965**	1.000	0.883*
Comet Tail Length	0.925**	0.883*	1.000
(d) **Low Dose Late**; N = 7			
Comet Tail DNA	1.000	0.971**	0.854*
Comet Tail Moment	0.971**	1.000	0.946**
Comet Tail Length	0.854*	0.946**	1.000
(e) **High Dose Early**; N = 8			
Comet Tail DNA	1.000	0.692	0.362
Comet Tail Moment	0.692	1.000	0.816*
Comet Tail Length	0.362	0.816*	1.000
(f) **High Dose Late**; N = 13			
Comet Tail DNA	1.000	0.929**	0.834**
Comet Tail Moment	0.929**	1.000	0.910**
Comet Tail Length	0.834**	0.910**	1.000

* Correlation value is significant at the 0.01 level (2-tailed)

** Correlation value is significant at the 0.05 level (2-tailed)

Table 2-9. Summary of univariate comparisons between the fixed effect factors herbicide treatment and time of exposure, and comet tail DNA (a), comet tail moment (b), and comet tail length (c), in isolated lymphocyte DNA of 7-day-old ducklings exposed *in ovo* to 2,4-D formulation spray.

(a) **Comet Tail DNA**	**Regression coefficient (β)**	**95% Confidence Intervals for β**		***P* value**
		Lower	**Upper**	
Herbicide Treatment				
- High Dose[1]	-0.007	-0.114	0.100	0.90
- Low Dose[2]	-0.021	-0.146	0.105	0.74
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.107	0.013	0.201	0.03*
- Late[5]	Reference	-	-	-
(b) **Comet Tail Moment**				
Herbicide Treatment				
- High Dose[1]	-0.012	-0.175	0.150	0.88
- Low[2]	-0.021	-0.211	0.170	0.83
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.138	-0.008	0.283	0.06
- Late[5]	Reference	-	-	-
(c) **Comet Tail Length**				
Herbicide Treatment				
- High Dose[1]	-0.006	-0.098	0.087	0.90
- Low Dose[2]	-0.046	-0.154	0.063	0.40
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.065	-0.022	0.151	0.14
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 21 of incubation

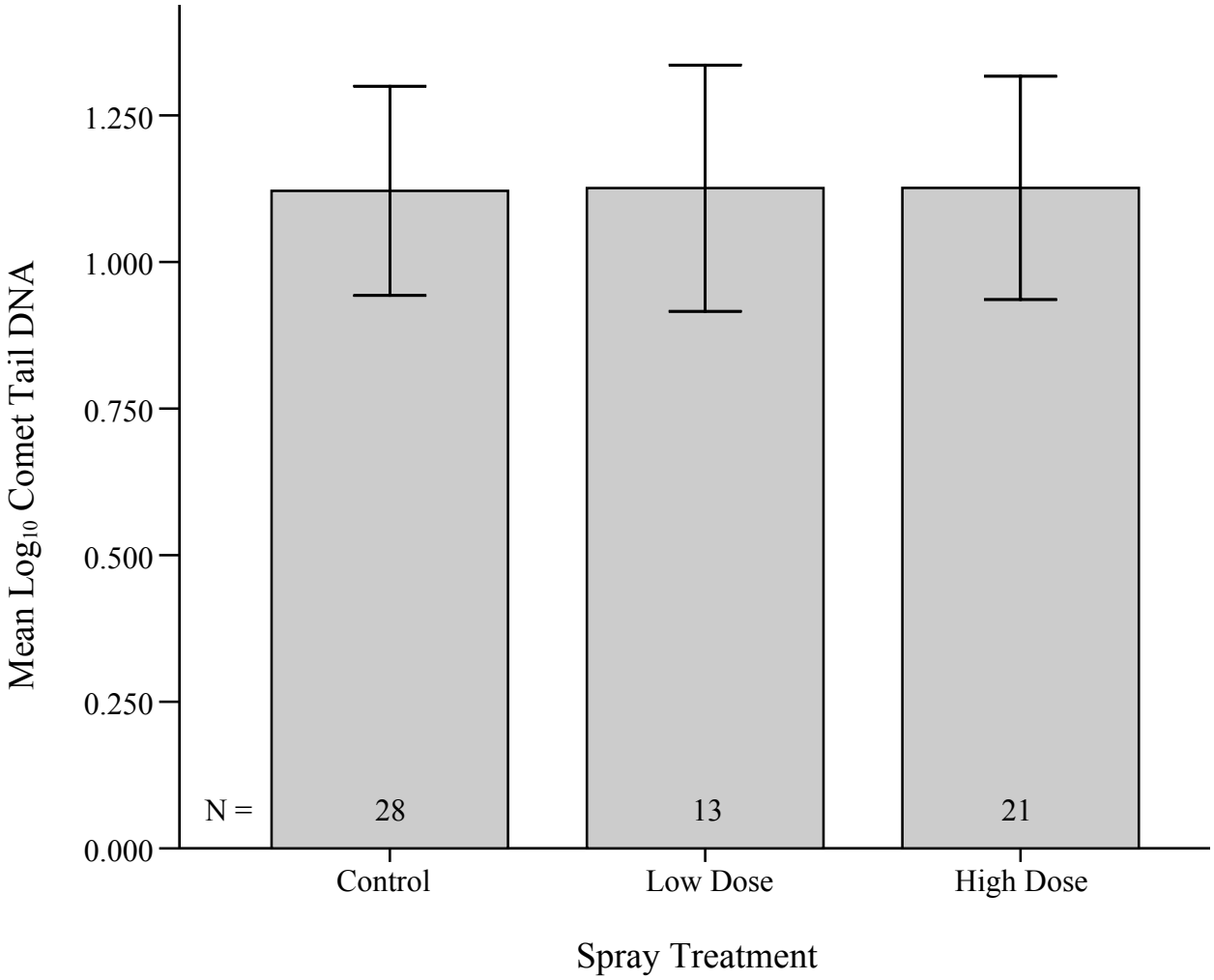

Figure 2-7. The simple association between comet tail DNA in domestic duck lymphocytes and *in ovo* 2,4-D herbicide exposure. The bars represent mean $\log_{10}$ comet tail DNA for the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD.

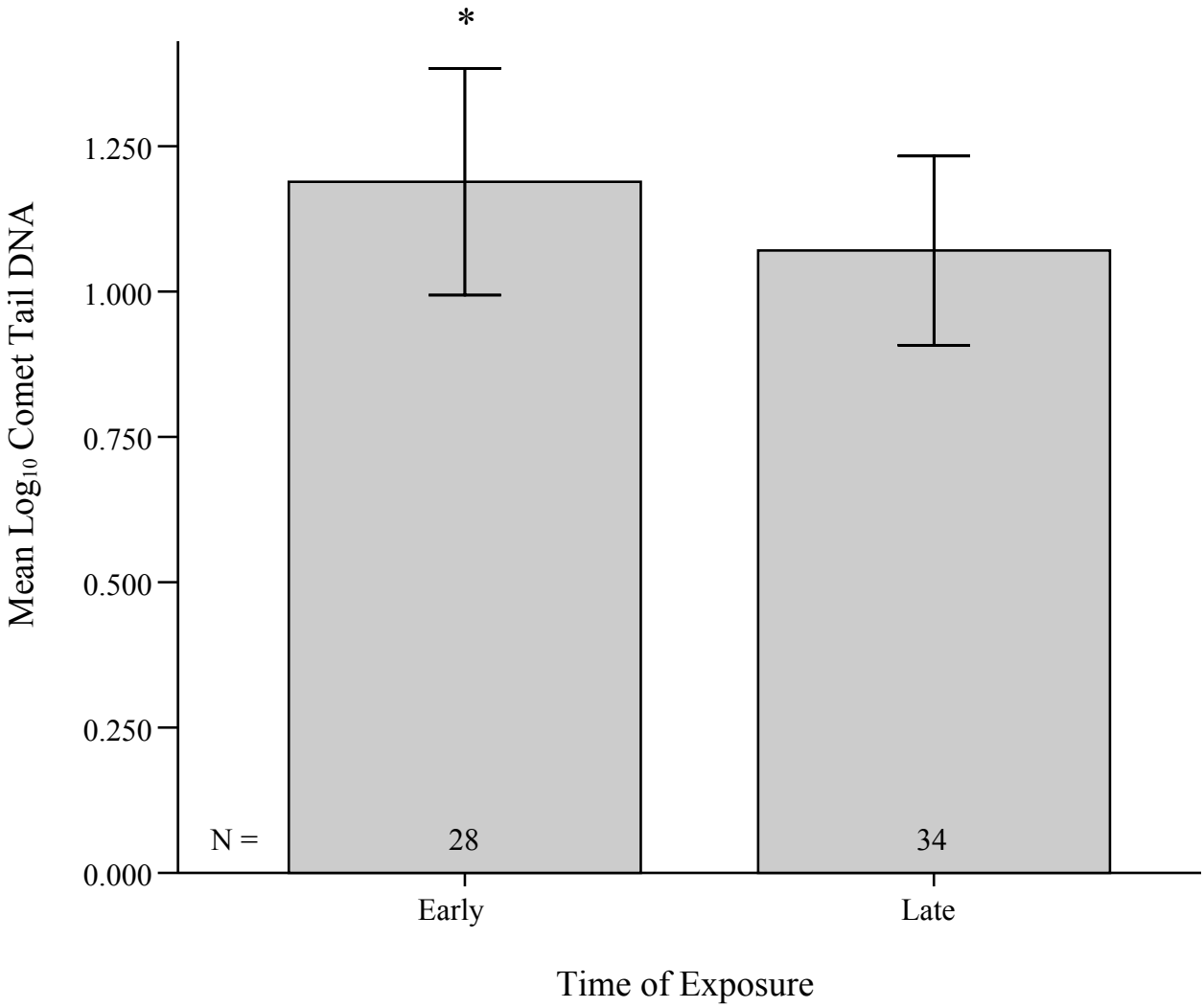

Figure 2-8. The simple association between comet tail DNA in domestic duck lymphocytes and the time of *in ovo* 2,4-D herbicide spray application. The bars represent mean $\log_{10}$ comet tail DNA for early (day 6 of incubation) and late (day 15 of incubation) exposure groups. Error bars represent ± SD of the mean. *Mean $\log_{10}$ tail DNA content was significantly different between early and late times of herbicide exposure ($P = 0.0269$).

Table 2-10. Descriptive summary of flow cytometry outcome, half-peak coefficient of variation (HPCV) in DNA content of circulating erythrocytes, from 21-day-old ducklings exposed *in ovo* to 2,4-D formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	19	3.93	0.42	0.10	3.90	3.70	4.10
	Late[2]	18	3.77	0.29	0.07	3.70	3.60	4.00
1 X 2,4-D (Low Dose)	Early[1]	9	3.68	0.68	0.23	3.60	3.10	4.35
	Late[2]	6	4.20	0.52	0.21	4.10	3.80	4.45
10 X 2,4-D (High Dose)	Early[1]	10	3.94	0.47	0.15	3.95	3.50	4.43
	Late[2]	22	4.03	0.37	0.08	4.05	3.70	4.30

[1] Day 6 and [2] Day 21 of incubation

Table 2-11. Summary of univariate comparisons between the fixed effect factors exposure time and herbicide treatment, and half-peak coefficient of variation (HPCV) in DNA content of circulating erythrocytes from 21-day-old ducklings exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	0.12	-0.07	0.32	0.22
- Low Dose[2]	0.02	-0.23	0.27	0.88
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.07	-0.26	0.12	0.44
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 21 of incubation

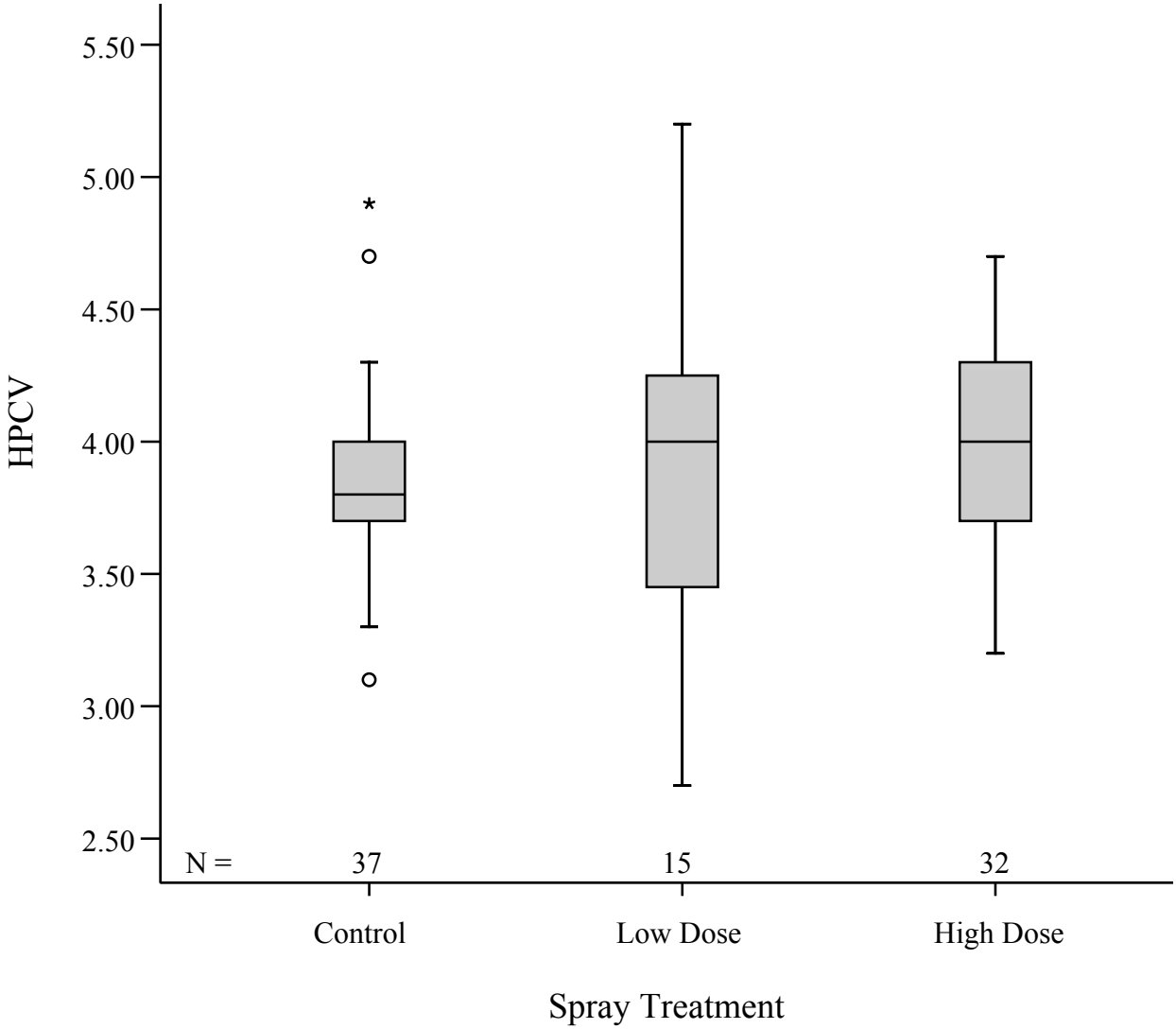

Figure 2-9. The simple association between flow cytometry outcome, half-peak coefficient of variation (HPCV) in DNA content of duck erythrocytes and *in ovo* 2,4-D herbicide exposure. Boxplots represent the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). The centre line represents the median, the box the interquartile range, the whiskers extend from the highest to lowest values excluding outliers. ○ and * represent outlier and extreme outlier values, respectively.

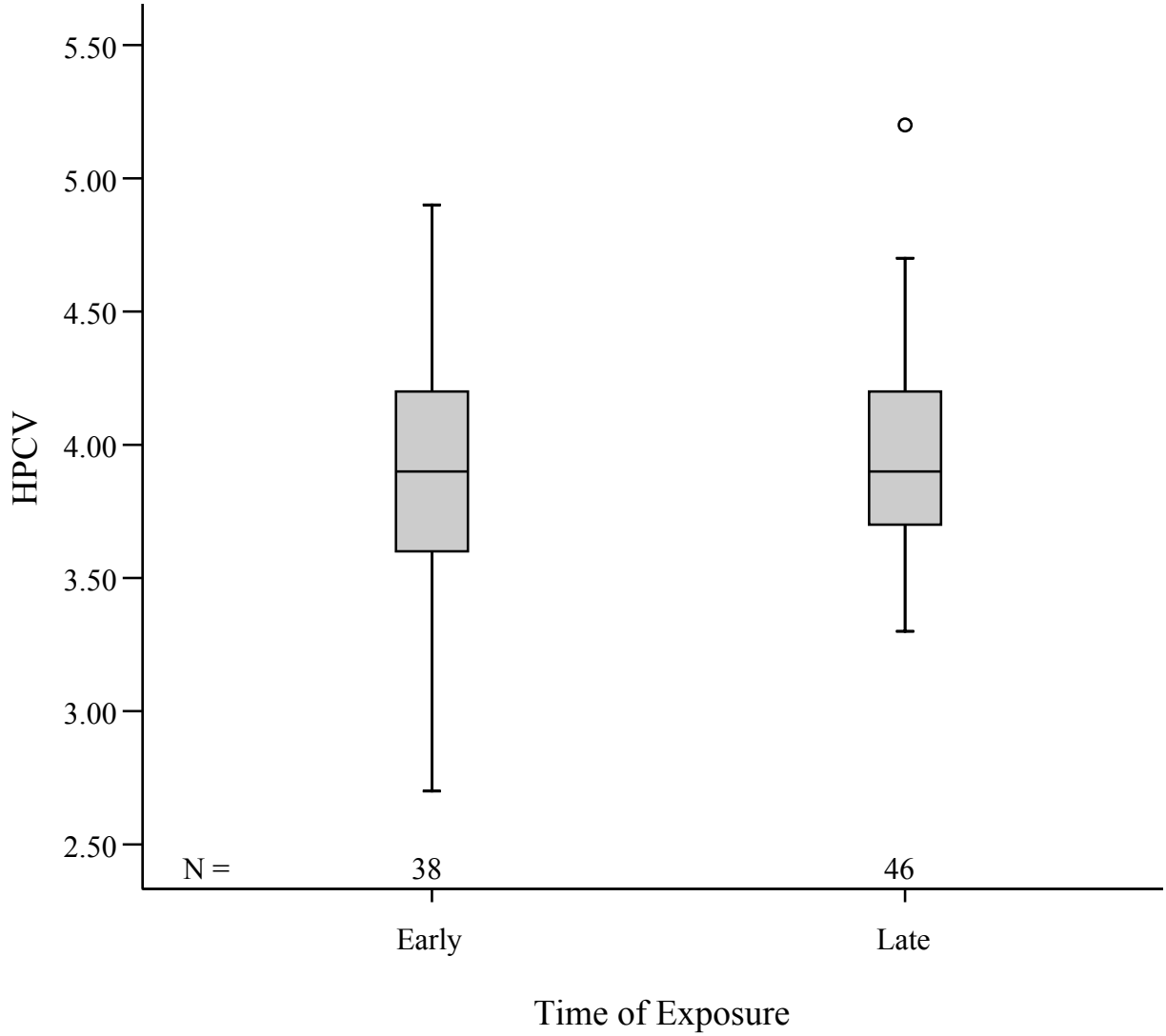

Figure 2-10. The simple association between flow cytometry outcome, half-peak coefficient of variation (HPCV) in DNA content of duck erythrocytes and the time of *in ovo* 2,4-D herbicide spray application. Boxplots represent the two times of herbicide exposure, early (day 6 of incubation) and late (day 21 of incubation). The centre line represents the median, the box the interquartile range, the whiskers extend from the highest to lowest values, excluding the outlier, shown as ○.

2.4 Discussion

Analysis of chicken and duck eggs demonstrated measurable transfer of herbicide residues through the shell and into the embryo by 24 hours after spraying. As expected, mean 2,4-D residue concentrations were higher in both chicken and duck eggs from the high dose (10X) groups than in eggs exposed to the recommended field rate of herbicide application (1X). Embryo residue concentrations in the chicken eggs (duck eggs not collected) increased from the day following exposure to 5 days after spraying, in both low and high dose groups. Mean concentrations in the 1X group increased from 0.6 to 2.2 ng/g, while 2,4-D residues in the 10X group increased from 27.4 to 374.5 ng/g during this time period. These findings are consistent with previous studies that have demonstrated the transfer of externally applied 2,4-D ester into bird embryos (Somers et al. 1974, Duffard et al. 1987, Castro de Cantarini et al. 1989, Várnagy 1999) and gradual uptake of the herbicide (subsequently increasing the amount of compound the embryo is exposed to) over the duration of embryonic development (Castro de Cantarini et al. 1989). The study performed by Castro de Cantarini et al. (1989) found that after fertile eggs were topically exposed to 2,4-D ester on E0, the herbicide was detectable in the embryo by E5 and continued to increase in concentration throughout embryonic development. This observation indicates that risk of contaminant-induced adverse effects may continue to increase for at least several days after exposure.

In ovo 2,4-D exposure in domestic chickens did not result in significant genotoxic effects, based on either endpoint examined. Differences in herbicide treatment (high and low concentrations) and times of exposure (early and late incubation stages) did not translate into noticeable factor effects in final model analyses for any assay variables. The comet assay metrics, percent DNA in comet tail, tail moment, and tail length, were all highly correlated. However, none of these comet assay endpoints to evaluate DNA strand breakage in isolated peripheral blood lymphocytes from herbicide treated chickens were statistically different than results from the control animals. In addition, in the flow cytometry assay, there was no treatment-related difference in the variability (measured by HPCV) of erythrocyte DNA content, and consequently no association between clastogenic damage and herbicide exposure.

Experiments using domestic ducks with 2,4-D exposures *in ovo*, resulted in no significant effects for DNA content analysis. Values of HPCV did not differ significantly among pesticide exposure groups, and between early and late exposed embryos. For the comet assay outcomes, time of exposure was a significant predictor for comet tail DNA content ($P = 0.03$). Time of exposure was almost an important factor for comet tail moment ($P = 0.06$), but not for comet tail length ($P = 0.14$), even though all variables were shown to be strongly correlated. Differences among these results for the three comet metrics may be explained by evidence which suggests that certain measurements of the comet tail are more sensitive indicators of genotoxicant exposure than others. It has been demonstrated that measurements of DNA migration (i.e. comet tail length) may be less accurate predictors of DNA damage than more quantitative measures of the amount of DNA strand breaks in the tail. Tail length tends to plateau at higher exposures (a limitation induced by electrophoresis conditions), while the amount of DNA in the tail can continue to increase (Fairbairn et al. 1995, Collins et al. 1997). Because it includes both intensity and migration, comet tail moment tends to be the most complete comet parameter. However, some researchers regard comet tail DNA content as the most appropriate measurement of genotoxicity, because tail DNA content is proportional to the number of DNA strand breaks, and it is able to discriminate up to complete damage (100%) in the tail (Collins 2004). In this study, all three variables were evaluated to provide a complete measurement of potential genetic damage.

If the amount of DNA in the tail is considered an important and discriminating variable for detecting genotoxicant exposure, then the observation that time of exposure had a significant effect on this variable for duck embryos may warrant further investigation. In agricultural areas of the Canadian prairies, wild bird eggs have the potential to be exposed to herbicide at any time after they are laid, so evaluation of sensitivity at two possible exposure times during incubation enhanced the environmental applicability of the study. There was no association between herbicide treatment and comet tail DNA content, therefore the significant difference observed in ducklings that were sprayed early in incubation from those birds exposed late in incubation cannot be attributed to 2,4-D treatment during these times. However, two distinct *in ovo* exposure timepoints were also tested because the vulnerability of the embryo may change with the stage of embryonic development (DeWitt et al. 2005) and exposure to spray conditions during early incubation may have contributed to differences in

comet tail DNA content. In chickens, embryonic day 6 (E6) represents a relatively late stage of organogenesis, while embryonic day 15 (E15) coincides with a period of later differentiation (Patten 1971). Duck eggs were sprayed at E6 and E21. The incubation period for ducks is usually 6-7 day longer than for chickens. E6 in ducks represents an earlier developmental stage than E6 in chickens, whereas E21 in ducks essentially matches physiological development at E15 in chickens. By spraying eggs at these timepoints, embryos may have experienced changes (temperature differences, external application of the spray to the egg, handling, etc.) during a potentially sensitive stage of development. The stress of the manipulations associated with egg handling and spraying at a vulnerable stage during incubation could be an issue. Since the ducks were sprayed at a relatively earlier embryonic period than the chickens, duck embryos were exposed to these conditions at a more critical stage of development or may be more vulnerable to this physical stressor than chickens at this timepoint. In chickens, the period from E0 up to E4 is the first important period for major organ formation and rapid tissue differentiation. Past this timepoint, negative effects on avian development can still occur, but embryos are particularly vulnerable up to and including E4 (DeWitt et al. 2005).

Lymphocytes from 7-day-old ducklings hatched from eggs that were experiences spray application at E6 showed increased amounts of DNA strand breaks compared with cells from eggs that were sprayed at E21. Strand breaks that remain unrepaired may lead to permanent genetic mutations, and this type of damage is considered an initiating point in the onset of carcinogenesis (Ponder 2001). Direct damage to DNA in germ cells may cause heritable mutations and teratogenic effects, and thus have the potential for greater impact at the population level (Mitchelmore and Chipman 1998). Over the years, the comet assay has been successfully used to identify environmental agents that cause an increase in DNA strand breaks in various cell types in numerous wildlife species. Although the genotoxicity of 2,4-D has been debated, the comet assay has been used in recent studies to show the potential of this herbicide to cause DNA strand breaks in mammalian and non-mammalian cells. In fish erythrocytes, a significant increase in comet tail length was observed after exposure to sub-lethal concentrations of 2,4-D (Ateeq et al. 2005). The genotoxicity of 2,4-D has also been demonstrated in Chinese Hamster ovary cells after exposure to the herbicide resulted in dose-dependant increases in the frequency of DNA strand breaks (González et al. 2005). The

present study was the first to assess the genotoxicity of an *in ovo* exposure to commercial 2,4-D formulation in avian embryos using the comet assay. Lymphocytes from 7-day-old ducklings that were exposed to spray early in incubation (E6) demonstrated increased DNA strand breaks ($P < 0.05$), but this association was not related to 2,4-D treatment. Spray exposure during later periods of embryonic development did not appear to affect genetic integrity in either domestic ducklings or chicks.

CHAPTER 3

IMMUNOTOXIC EFFECTS OF *IN OVO* 2,4-D EXPOSURE IN DOMESTIC CHICKENS (*GALLUS GALLUS*) AND DUCKS (*ANAS PLATYRHYNCHOS*)

Abstract

Reduced soil tillage and winter cereal seeding are commonly used farming techniques on the Canadian prairies. These practices result in increased crop cover in the spring, thus providing attractive habitat for ground nesting birds, such as upland game birds and waterfowl. The nesting period for these species often coincides with herbicide treatment of many important cereal crops. Therefore, eggs of ground nesting birds have the potential to be exposed during routine spray applications. Among the most commonly used herbicides for early broadleaf weed control on the Canadian prairies is 2,4-dichlorophenoxy acetic acid (2,4-D). Research has shown that 2,4-D is immunotoxic or has the potential to alter immune function in laboratory animals. However, knowledge of the potential effects of 2,4-D on the immune function of young birds is inadequate to assess realistic ecotoxicological concerns of contaminant exposure. The present study assessed the effects of *in ovo* exposure to a 2,4-D ester herbicide formulation on the immune system of newly hatched domestic chickens (*Gallus gallus*) and ducks (*Anas platyrhynchos*).

Fertile eggs of both species were sprayed with the herbicide at either field application rates, or at 10 times recommended rates, on days 6 or 15 (chickens) and 6 or 21 (ducks) of incubation, to evaluate risks during early or late developmental stages, respectively. Control groups consisted of eggs sprayed with water only. The potential immunotoxic properties of 2,4-D were assessed using standard assays to evaluate cell-mediated immunity, humoral immune function and immunopathology in hatchlings. The cell-mediated immune response was measured using a delayed-type hypersensitivity (DTH) reaction to bovine serum albumin (BSA) in 21-day-old birds, and humoral immune function was assessed by measuring systemic antibody production (via an enzyme-linked immunosorbent assay (ELISA)) following BSA immunization. Additional endpoints evaluated included differential white

blood cell counts to determine heterophil/lymphocyte (H/L) ratios, relative lymphoid organ weights and histopathology of immune organs.

Potential associations between herbicide exposure and the immunotoxicity endpoints were analyzed using a mixed linear statistical model. Herbicide treatment and time of exposure were accounted for as fixed factors. Relatively few significant associations were observed among the fixed effect factors for the general immune assessment outcomes. The relative weight of the bursa of Fabricius in chickens ($P = 0.0006$), and the H/L ratio in ducks ($P = 0.04$) demonstrated significant relationships with herbicide treatment. Bursal weight in 21-day-old chicks exposed to low dose herbicide application decreased compared to the birds in the control groups, while the H/L ratio increased in ducklings of the same age with exposure to 2,4-D. With time of spray exposure as a factor, the only significant association observed was with relative bursal weight in ducks ($P = 0.04$). Bursal weight in 21-day-old ducklings decreased when eggs were sprayed at the later period in incubation. However, there was no significant association between 2,4-D treatment and bursal weight in ducklings, therefore differences in weights between times of exposure cannot be attributed to effects of 2,4-D. Results of both functional assays (DTH and antibody production measured with ELISA) provided no evidence that *in ovo* exposure to commercial 2,4-D herbicide formulation, at the incubation stages and application rates used, affected immune function in young chickens and ducks.

3.1 Introduction

Farming practices used during the past 15 to 20 years have reduced the impact of agriculture on the environment through reduced tillage and use of fall planted (winter cereal) crops. These strategies minimize degradation of soil, water, and air quality, as well as maintain wildlife habitat and biodiversity on the prairies, because implementation of these techniques generally results in increased vegetative ground cover in the spring. Farmland that receives reduced till and/or winter cereal seeding usually provides superior habitat for upland wildlife species, including ground-nesting birds. However, these techniques also generally require weed management practices which may increase herbicide exposure of nesting birds and their young.

Collectively, farmers in the prairie provinces are more likely to apply pesticides to their crops than farmers in other parts of Canada (Boame 2005). Spring herbicide application

is especially important for weed management on farmland receiving minimal till and in fields seeded with winter cereal crops, since it is the major alternative to tillage (Korol 2004). Herbicide application rates are typically higher in low or no till fields, because cultivation operations are often replaced with intensified herbicide application to control weeds (Gebhardt 1985). 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most commonly used herbicide in prairie agriculture (Fowler 2002). Because spraying periods for spring weed control overlap with the nesting period of many species of ground-nesting waterfowl and upland game birds, the risk of egg exposure to 2,4-D is significant. The potential long-term effects of low rates of exposure to 2,4-D in wildlife, including avian species, is poorly understood, especially when exposure occurs during embryonic development. Therefore, because conservation practices may increase the risk of *in ovo* exposure to 2,4-D in critical prairie breeding areas, it is important to investigate potential sublethal effects of this herbicide on developing birds.

The immature and early life stages of mammalian and non-mammalian species are the most vulnerable to immunomodulation by chemicals introduced into the environment. Immune dysfunction can result from alterations during development, and these effects may be long-term, or not expressed until later in life. Immunomodulating effects of environmental contaminants on specific immune responses can be used as sensitive biomarkers of toxicant exposure (Keller et al. 2000, Grasman 2002). Numerous studies have been conducted in recent years to investigate the effects of environmental contaminants on immune function in domesticated and wild birds. The effects of metals, polychlorinated biphenyls (PCBs), pesticides, and organochlorine compounds have been evaluated in a variety of avian models, including chickens (*Gallus* spp.) (Knowles and Donaldson 1997, Lee et al. 2001, Lee et al. 2002, Finkelstein et al. 2003, Singhal et al. 2003), mallard ducks (*Anas platyrhynchos*) (Fairbrother and Fowles 1990, Fowles et al. 1997), avocets (*Recurvirostra americana*) (Fairbrother et al. 1994) western bluebirds (*Sialia mexicana*) (Fair and Myers 2002), tree swallows (*Tachycineta bicolor*) (Bishop et al. 1998), American kestrels (*Falco sparverius*) (Smits and Bortolotti 2001), Japanese quail (*Coturnix japonica*) (Grasman and Scanlon 1995), gulls (*Larus* spp.) (Grasman et al. 1996, Bustnes et al. 2004), and Caspian terns (*Sterna caspia*) (Grasman et al. 1996, Grasman and Fox 2001). Several studies have assessed the effects of *in ovo* contaminant exposure in birds, and evaluated

certain aspects of the immune response after introducing the contaminant at precise developmental stages (Fairbrother et al. 1994, Bunn et al. 2000, Lee et al. 2001, Lee et al. 2002, Singhal et al. 2003). One study, evaluated immunotoxic effects of *in ovo* herbicide exposure in Northern bobwhite (*Colinus virginianus*) chicks. Dabbert et al. (1997) assessed the immunocompetence of chicks exposed *in ovo* to clopyralid (3,6-dichloro-2-pyridinecarboxylic acid) spray treatment at field application rates.

2,4-dichlorophenoxy acetic acid, has been widely used throughout the world since the 1950s. Although numerous studies have evaluated its toxicity, including its effects on the immune system, the effects of 2,4-D on many physiological functions are unclear or appear contradictory. Exposure in mice causes a decrease of serum antibody titres (Salazar et al. 2005). This evidence suggests that 2,4-D could potentially have long-term effects on the humoral immune response. However, research on the immunotoxicity of 2,4-D is inconclusive. Acute oral exposure to 2,4-D increased the number of antibody-producing cells in spleens of mice (Blakley 1986), while acute dermal exposure suppressed antibody production in the same species (Blakley and Schiefer 1986). Oral 2,4-D treatment did not have an effect on antibody production or other immune functions in Fisher rats (Blakley et al. 1998). When the toxicity of different herbicide mixtures of 2,4-D were evaluated in mice, results indicated immunosuppressive effects. For example, oral exposure to a mixture of 2,4-D and the herbicide picloram resulted in reduced antibody production in spleen cells (Blakley 1997), and intraperitoneal injection of a mixture of 2,4-D and 3,4-dichloropropionanilide (propanil) caused decreased thymocyte populations and thymic weight (de la Rosa 2005). These studies with laboratory animals have shown that 2,4-D may be immunotoxic. However, little is known about the subtle effects of this herbicide on the immune function of birds, especially when exposure occurs during embryonic development.

This study was performed to investigate whether *in ovo* exposure to a commercial 2,4-D herbicide spray formulation was associated with changes to the immune function of domestic chickens (*Gallus gallus*) and ducks (*Anas platyrhynchos*), as surrogates for upland game birds and wild waterfowl, respectively. The potential immunotoxic properties of 2,4-D were assessed using standard assays to evaluate cell-mediated immunity, humoral immune function and immunopathology in hatchlings.

The delayed-type hypersensitivity (DTH) test has been used successfully to assess modulation of cell-mediated immune function in birds exposed to environmental contaminants. In mallards (*Anas platyrhynchos*), a relationship was found between a decreasing T cell inflammatory response in the DTH test and increasing selenomethionine dose (Fairbrother and Fowles 1990), while *in ovo* exposure to lead was associated with depressed DTH response in chickens (*Gallus gallus*) (Lee et al. 2001, Lee et al. 2002). In the present study, the cell-mediated immune response in 21-day-old birds exposed *in ovo* to 2,4-D was evaluated using the DTH test following sensitization with bovine serum albumin (BSA).

One valuable test to evaluate immune function is to measure the strength of the humoral (antibody-mediated) immune response following antigen exposure. The ability of an individual to produce antigen-specific antibodies is measured by using the enzyme-linked immunosorbent assay (ELISA) (Smits and Janz 2005). Several ecotoxicological studies have used the ELISA technique to detect specific immunoglobulin levels in wild birds. Using a standard ELISA method, Bustnes et al. (2004) found that female glaucous gulls (*Larus hyperboreus*) with high blood concentrations of organochlorine pesticides showed a decreased immune response to novel antigen immunization. The ELISA has also been used to measure antibody responses in western bluebird (*Sialia mexicana*) nestlings exposed to lead shot (Fair and Myers 2002) and in American kestrels (*Falco sparverius*) exposed to polychlorinated biphenyls (Smits and Bortolotti 2001). In studies with domestic chickens, the ELISA has been used to measure antigen-specific immunoglobulin levels in birds exposed *in ovo* to pesticides (Singhal et al. 2003) and lead (Lee et al. 2002).

Another accepted method of assessing the effect of xenobiotics on the immune system is to examine the characteristics of associated cells, tissues, and organs in exposed animals. Sampling and studying these components, termed immunopathology, provides general information about immune structure (Keller et al. 2000). The present investigation evaluated the health of the immune system using various tests, including differential white blood cell counts, to measure relative number of heterophils and lymphocytes, and examination of immune organ weights and histology to assess immune organ structure.

The design of the *in ovo* herbicide exposure was intended to simulate a scenario in which eggs of ground nesting waterfowl or upland game birds are sprayed with herbicide

during weed control operations. The results of this study will help to determine the subtle impacts of 2,4-D on immune health following exposure during different stages of avian embryonic development, using domestic chicks and ducklings as models.

3.2 Materials and Methods

3.2.1 Animal Model

Fertile chicken (*Gallus gallus*) eggs from a White Leghorn/Brown Leghorn cross were incubated at 37.5°C and approximately 80% humidity in circulated air incubators (Hova-Bator®, G.Q.F. Manufacturing Co., Savannah, Georgia, USA) until 1 day post-hatch (about day 23). Automatic egg turners (Hova-Bator®, G.Q.F. Manufacturing Co., Savannah, Georgia, USA) were used for the first 18 days of incubation. On day 19 of incubation, egg turners were removed and eggs were placed on the wire floor of the incubator. Humidity was increased to approximately 90% in accordance with hatching requirements. Chicks were transferred to heated brooders with raised wire floors at one to two day(s) of age, and maintained on *ad libitum* chick starter and fresh water for the duration of the study. Fertile Pekin duck eggs, a domestic mallard breed (*Anas platyrhynchos*), were raised in a similar fashion with the following modifications. Duck eggs were incubated at > 90% humidity for approximately 28 days until hatch, then transferred to dry incubators until 1 day post-hatch. Automatic egg turners were used for the first 25 days of incubation, and on day 26 egg turners were removed. Ducklings were transferred to pens with solid heated floors bedded with aspen shavings or straw for the remainder of the study.

3.2.2 Herbicide Spray Exposures

Fertile chicken and duck eggs (3 replicate groups each of 120 eggs) were randomly assigned to one of six incubators (20 per incubator), and each incubator was randomly assigned to a specific treatment. Different types of developmental effects may be attributed to immune system insult during specific periods of embryonic development. Therefore, in order to account for time-specific vulnerability of the embryos, eggs were exposed to the herbicide during either an early (day 6 for chickens and ducks) or late (day 15 for chickens and day 21 for ducks) stage of incubation (DeWitt et al. 2005).

Eggs were sprayed with a commercial formulation of 2,4-D ester (Interprovincial Cooperative Limited Agri Products Department, Winnipeg, Manitoba, Canada) at one of two different concentrations: 1) Low dose groups (both early and late

exposure times) were sprayed with 2,4-D at the field application rate recommended for winter wheat (0.56 L ai ha^{-1}) (SAFRR 2005). This was equivalent to 4.24 ml of 2,4-D per litre of herbicide solution. 2) High dose groups (early and late) were sprayed with 42.40 ml 2,4-D/L, equivalent to 10 times the recommended concentration of herbicide, to simulate a worst-case exposure. Additional groups of eggs (early and late) were sprayed at the same time points with water only, to act as negative control groups. The spray treatments were applied using an agricultural field spray simulator (Research Instrument Company, Guelph, Ontario, Canada) (Figure 2-1) at the Agriculture and Agri-Foods Canada Research Centre in Saskatoon, Saskatchewan, to reproduce actual field application conditions as closely as possible. Eggs were masked prior to spraying to ensure that every egg received similar amounts of herbicide (Figure 2-1). The six treatment groups comprising each replicate were: high 2,4-D dose (early and late incubation exposures); low 2,4-D dose (early and late); and negative control (water only, early and late). The three replicates, for both chicken and duck experiments, were spaced about two weeks apart.

3.2.2.1 Quantifying Herbicide Exposure

Herbicide deposition on the surface of the eggs was quantified in a separate study by spraying surplus eggs of both species with fluorescein dye solution (10% (w/w) fluorescein sodium salt in water) at the same application rate as the herbicide treatments. The amount of fluorescein dye deposited on the exposed portion of the eggs was determined by rinsing the eggs to remove the dye, and determining the amount of fluorescein in the rinsate. The rinsate fluorescence was measured at 498 nm using a spectrofluorometer (Shimadzu RF-1501, Shimadzu Corporation, Columbia, MD, USA).

Surplus chicken and duck eggs were collected at 1 and 5 (chickens only) days after spraying on day 6 of incubation, for analysis of 2,4-D residue concentrations in the embryos. After extraction from the shell on the side opposite herbicide deposition, embryo samples were homogenized (Brinkmann POLYTRON® homogenizer, Brinkmann Instruments, Inc., Westbury, New York, USA) and 2,4-D residues were extracted with acetonitrile (Caledon Laboratories Ltd, Edmonton, Alberta, Canada). Herbicide concentrations in embryo extracts were measured with a high performance liquid chromatograph (LC) (Waters 2695 Alliance System, Milford, Massachusetts, USA) coupled with tandem mass spectrometry (MS/MS) (Waters Micromass® Quattro Ultima™, Milford, Massachusetts, USA). Concentrations of

2,4-D (ng/ml) were determined using a known amount of internal standard (deuterated 2,4-D (d5), Cambridge Isotope Laboratories, Cambridge, Massachusetts, USA), and corrected to compensate for losses associated with sample processing during the extraction procedure (50%) and for extraction efficiency (50%) of the LC-MS/MS.

3.2.3 Sample Collection

Data for immunoassays were collected at four different times post hatching. For the antibody response measured with the ELISA, blood was collected from all 7-day-old birds to determine baseline serum antibody titres, and birds were subsequently immunized with BSA. On day 14, blood was collected to determine the primary antibody response to the initial immunization, and birds were immunized again with BSA. For the DTH test, pre-exposure wing web measurements were obtained on day 20, followed by intradermal wing web injections with BSA. On day 21, post-exposure wing web measurements were taken, and blood was again collected to determine the secondary antibody response to BSA immunization, as well as for differential white blood cell counts. All of the birds were euthanized following blood collection on day 21, and selected immune organs (thymus, spleen, bursa of Fabricius) were collected, weighed (with the exception of the thymus) and preserved for histopathological examination.

The ELISA was performed on serum samples collected from 7-day-old (baseline antibody titres), 14-day-old (primary response), and 21-day-old (secondary response) birds immunized with BSA. Birds were immunized on day 7 and again on day 14 by injecting 0.5 ml of BSA at 4 mg/ml in physiological, sterile saline subcutaneously into one site on the dorsal scapular region. Blood samples were collected from the jugular vein with a heparinized syringe into Eppendorf® microcentrifuge tubes, and kept on ice until further sample processing was performed. Within 6 hours of blood collection, samples were centrifuged for 10 min at 5000 rpm (Eppendorf Centrifuge 5415D, Brinkmann Instruments Inc., Westbury, NY, USA). Serum was carefully withdrawn from the tubes with a pipette, transferred into low temperature freezer vials, and stored at -80°C until the ELISA was performed. Unless otherwise indicated, all chemicals and reagents were obtained from Sigma Aldrich Canada Ltd. (Oakville, ON), and laboratory supplies and disposables were purchased from VWR International (Mississauga, ON).

3.2.4 ELISA Protocol for Detection of Specific IgG Antibodies

Humoral immune function was evaluated using a modified ELISA technique (Smits and Bortolotti 2001) to measure IgG class antibody titres in the blood serum of birds immunized with BSA. Microtiter plates (96-well, flat bottom, Nunc-brand, Nalgene) were coated with 100 µl/well BSA at 0.5 µg/ml in carbonate coating buffer (pH 9.6) and incubated at 4˚C for 15 hours. Following incubation, plates were rinsed 4X with 0.05% phosphate buffered saline-Tween 20 (PBS-T, pH 7.3), and residual binding sites blocked with 5% (reconstituted) dried skim milk (100 µl/well) for one hour at room temperature. Plates were rinsed 4X with PBS-T after blocking. Serum samples and standards were diluted in PBS-T. Positive and negative controls consisted of pooled serum from day 21 and day 7 (pre-sensitized) birds, respectively. Twofold dilutions of sera (100 µl/well), beginning with a dilution of 1/50 for chickens or 1/1 for ducks, were added to duplicate rows across the plates, followed by incubation at room temperature for two hours. Plates were rinsed 4X with PBS-T and 100 µl of rabbit anti-chicken IgG (1:400, Bethyl Laboratories, Inc., Montgomery, TX, USA) or goat anti-duck IgG (1:200, KPL, Gaithersburg, MD, USA) was added to each well, followed by incubation at room temperature for one hour. Plates were then rinsed 4X with PBS-T, and 100 µl of goat anti-rabbit (1:800, Bethyl Laboratories, Inc., Montgomery, TX, USA) or rabbit anti-goat (1:400, KPL, Gaithersburg, MD, USA) horseradish peroxidase conjugate was added to each well, followed by incubation at room temperature for 1 hour. Plates were again rinsed 4X with PBS-T, and 100 µl of ABTS® horseradish peroxidase substrate (2,2'-azino-di (3-ethyl-benzthiazoline-6-sulfonate) in glycine/citric acid buffer, KPL, Gaithersburg, MD, USA) was added to all wells, and the plates were incubated in the dark at room temperature for 12 minutes. Finally, 100 µl of stop solution (1% SDS) was added to all wells, and absorbance at 405 nm was measured using a microplate spectrophotometer (SPECTRAmax® 190, Molecular Devices Corporation, Sunnyvale, CA, USA) with SOFTmax® PRO software (version 4.0, Molecular Devices Corporation, Sunnyvale, CA, USA). Anti-BSA antibody titres for chicks and ducklings exposed *in ovo* to 2,4-D are the reciprocal of the highest dilution of serum with an optical density value greater than the cutoff value. The cutoff value was the mean optical density value for the pooled negative control serum sample (containing baseline antibody levels). Statistical analysis was performed on the antibody titre (log transformed to attain normality) for samples collected

from 14- and 21-day-old birds. These values reflect the strength of the primary and secondary humoral response, respectively.

3.2.5 Delayed-type hypersensitivity (DTH) Test for T cell Response

Sensitization with BSA for stimulating a humoral immune response also acted as antigen sensitization for the DTH test. This test was conducted on 20-day-old chicks and ducklings. The right wing web of each bird was plucked free of feathers, marked to identify the injection site, and the thickness of the wing web at that spot was measured to the nearest 0.01 mm using a spring-loaded dial micrometer (Mitutoyo, Precision Graphic Instruments, Spokane, WA, USA). Three measurements were taken of the same site, and the mean value was recorded. The marked site was swabbed with 70% ethanol and injected intradermally with 0.1 ml of BSA solution (20 mg/ml in saline) using a 27-g needle. The thickness of the injection site was re-measured 24 hrs later by the same operator using the same technique, and the DTH response was reported as the difference in wing web thickness (Smits et al. 1999) using the following formula:

$$\frac{\text{Mean thickness of wing web (post-injection)} - \text{Mean thickness of wing web (pre-injection)}}{\text{Mean thickness of wing web (post-injection)}}$$

3.2.6 Hematology – Differential Leukocyte Count

Blood smears (two per bird) prepared from samples collected on day 21 were air dried and stained with Diff-Quik® (Dade Behring Inc., Newark, DL, USA). The ratio of heterophils to lymphocytes in peripheral blood was determined for each bird by counting 100 leukocytes per slide at 400X total magnification. The ratio of heterophils to lymphocytes is a useful indicator of stress in some avian species (Gross and Siegel 1983, Grasman et al. 1996, Maxwell and Robertson 1998).

3.2.7 Relative Organ Weights and Histopathology

On day 21, birds were weighed (± 0.01 g), bled and euthanized by cervical dislocation in accordance with protocols approved by the Canadian Council on Animal Care. Selected lymphoid organs (thymus, spleen, and bursa of Fabricius) were collected and fixed in 10% neutral buffered formalin within 15 min of death. Prior to fixation, the spleen and bursa of Fabricius were trimmed of adherent fat and connective tissue, and the mass of each organ determined (± 0.01 g) to calculate the relative organ weight, or somatic index (somatic index = [organ weight/body weight – organ weight] x 100).

For histopathological examination, cross-sections of two thymic lobes, and the spleen and bursa of Fabricius were embedded in paraffin, routinely processed, stained with hematoxylin and eosin, and examined by a veterinary pathologist. Cross-sections of all three organs were examined for evidence of overt pathology, including lymphocyte depletion or lymphoid atrophy. The organs were evaluated on the basis of organ-specific criteria, and subjectively compared between control and treatment groups. In the thymus, the relative thickness of the cortex and medulla was compared. In the spleen, the relative proportion of the white and red matter was evaluated. In the bursa of Fabricius, the size of the lymphoid follicles and the follicular cortico-medullary ratio were compared between control and treatment groups. Rates of mitoses and apoptoses in the bursa and thymus were also compared between treatments as a subjective measure of organ status.

3.2.8 Statistical Analysis

Descriptive statistics for all outcomes were compiled using SPSS (v.14.0, SPSS Inc., Chicago, IL, USA) and SYSTAT (v.11.0, SPSS Inc., Chicago, IL, USA). Summaries are reported for the subset of animals used for determining antibody response with the ELISA (N=190 for chickens, N=84 for ducks), the DTH test (N=85 for chickens, N=85 for ducks), the relative immune organ weights (N=129 for chickens, N=85 for ducks), and the assessment of heterophil/lymphocyte ratios (N=199 for chickens, N=85 for ducks). The normal distribution of all assay variables was assessed using the Shapiro-Wilk test for normality. All parameters were $\log_{10}$ transformed to attain normality if the p-value given by this test was low (i.e. < 0.5).

The association between exposure and immune endpoints was analyzed using a mixed linear model (PROC MIXED in SAS v.8.2 for Windows, SAS Institute, Cary, NC, USA). Incubator and experimental group were included as random effects to account for clustering of the observations as a result of separate incubator designations and replicated experiments, respectively. First, time of exposure (early or late incubation stage) and then herbicide treatment level (high or low concentration or water control) were assessed as fixed effect factors in a model including the random effects. Where time of exposure and herbicide treatment were both potentially important factors ($P < 0.25$), both were included in a model to assess confounding effects. Variables were retained in the final model if they were significant ($P < 0.05$), or acted as important confounders (i.e. adjustment for the variable

changed the other coefficient by more than 10%). If both time of exposure and herbicide treatment were significant ($P < 0.05$), the model was then tested for interaction.

3.3 Results

Herbicide deposition on the surface of the eggs was quantified in a separate study in which fluorescein dye was sprayed onto masked eggs, eggs were rinsed, and the amount of fluorescein in the rinsate was measured using a spectrofluorometer. Mean doses of the 2,4-D active ingredient deposited onto masked eggs were calculated for low and high dose exposure groups, and reported in Table 2-1.

2,4-D was detected in chicken and duck embryos collected 1 and 5 (chickens only) days after early (day 6 of incubation) *in ovo* spray exposure. Herbicide residue concentrations in embryos from both treatment levels were determined using LC-MS/MS analysis of egg extracts (N = 3). In chickens, eggs from both high and low dose herbicide treatment groups contained measurable 2,4-D concentrations at 1 (embryonic day 7, E7) and 5 (E11) days after spraying. Embryo 2,4-D concentrations from eggs treated with the low dose concentration increased from a mean of 0.6 ng/g at stage E7 to 2.2 ng/g in eggs collected on E11. A similar trend was observed in chicken eggs treated with the high concentration of 2,4-D, with embryo residue concentrations increasing from 27.4 ng/g at E7 to 374.5 at E11. As expected, higher 2,4-D concentrations were observed in eggs treated at the 10X rate than in those treated with the recommended field application rate (1X). Duck eggs treated with 2,4-D at the 1X application rate contained a mean herbicide concentration of 2.46 ng/g, while eggs sprayed with 10X 2,4-D had 14.1 ng/g.

Descriptive statistics for the measurements of serum antibody concentrations in chickens following BSA immunization are summarized in Table 3-1. Serum samples from both 14-day-old chicks (post-BSA primary immunization), and 21-day-old chicks (post-BSA secondary immunization) contained higher concentrations of anti-BSA antibodies (positive response against BSA at a higher dilution) than pre-immunization sera, indicating that birds responded as expected to BSA immunization. Antibody concentrations were broadly similar to controls and 2,4-D treated birds (for both times of exposure), suggesting that neither herbicide treatment nor timing of spray exposure affected the humoral immune response of these birds.

Table 3-2 summarizes the univariate comparisons between the fixed effect factors, herbicide treatment and time of exposure, and antibody production as measured by ELISA. For 21-day-old chicks, only herbicide treatment ($P = 0.13$) was considered a potentially important factor in influencing antibody response ($P < 0.25$), and in the final model, there were no differences for either fixed effect factor when herbicide exposed groups were compared to the control groups. With herbicide treatment as a factor, mean sera dilution values for the low dose herbicide treatment was significantly different than the high dose treatment values ($P = 0.03$). However, neither the low dose nor high dose sera dilution values were different from the control group, therefore the observed difference in antibody response between the two herbicide treatment groups was deemed unimportant. For the 14-day-old chicks, herbicide treatment and time of exposure were not considered to be important factors in the initial model ($P > 0.25$). Figure 3-1 shows the relationship between all sera dilutions and the fixed effect factor herbicide treatment. Figure 3-2 shows that the fixed effect factor time of exposure did not have an effect on the humoral immune response as measured by antibody production following immunization with BSA.

A descriptive summary of the antibody response to BSA immunization in ducks is presented in Table 3-3. As in chickens, post-BSA immunization sera in ducklings contained higher concentrations of anti-BSA antibodies than pre-immunization sera, demonstrating a positive humoral response to antigen exposure. Table 3-4 summarizes the univariate comparisons between the fixed effect factors herbicide treatment and time of exposure, and antibody production as measured by ELISA. For sera dilutions of 14- and 21- day old ducks, herbicide treatment and exposure time were not important factors in the initial model ($P > 0.25$). Figures 3-3 and 3-4 illustrate the lack of significant relationship between the fixed factors herbicide treatment and exposure time and all sera dilutions (mean values).

Descriptive statistics for the outcomes from the DTH test in chickens are summarized in Table 3-5. The DTH response was assessed by measuring the change in thickness of the wing web after intradermal injection of BSA. Simple comparisons of the test outcomes from the herbicide treated birds to those of the control groups revealed no differences. However, there were differences observed between the low and high dose herbicide treatment groups. Table 3-6 summarizes the univariate comparisons between the fixed effect factors herbicide treatment and time of spray exposure, and the mean differences in wing web thickness.

Herbicide treatment was not associated with the ability of birds to mount a DTH response when low ($P = 0.21$) and high ($P = 0.71$) dose groups were compared to the negative control group. However, when herbicide treatments were compared to each other, a significant difference was observed ($P = 0.04$). Although statistically different, the biological significance of this observation is unclear. Time of exposure was not considered to be an important factor in the final model ($P = 0.78$). Figure 3-5 illustrates the relationship between mean differences in wing web induration and herbicide treatment, while Figure 3-6 demonstrates the lack of a significant relationship between the fixed effect factor exposure time and the DTH response in chickens.

The descriptive summary of the DTH response in 21-day-old ducklings is presented in Table 3-7. Simple comparisons of the test outcomes from herbicide treated birds to those of the control group revealed no differences. This result was reaffirmed when the effects of each factor was tested using univariate model analysis (Table 3-8). The factors herbicide treatment ($P = 0.27$) and time of exposure ($P = 0.48$) were not considered to be important factors in the initial statistical model, nor did they have significant effects on the DTH response in the final model. Figures 3-7 and 3-8 show the relationships between the mean DTH response and each fixed effect factor.

Descriptive statistics for the ratio of heterophils to lymphocytes in peripheral blood samples from 21-day-old chicks are summarized in Table 3-9. Table 3-10 summarizes the univariate comparisions between the mean ratios and both fixed effect factors. Herbicide treatment ($P = 0.84$) and time of exposure ($P = 0.32$) did not have a significant effect on the numbers of heterophils and lymphocytes in whole blood samples. Figures 3-9 and 3-10 demonstrate that neither of the fixed effect factors had a significant effect on H/L ratios in chickens.

Descriptive statistics of the H/L ratios in peripheral blood samples from 21-day-old ducklings are summarized in Table 3-11. Simple comparison of mean ratios indicates no differences between herbicide treatments and/or times of exposure and the ratio of heterophils to lymphocytes. Univariate comparisons from the mixed linear analysis model are summarized in Table 3-12. In the final model, herbicide treatment was associated with H/L ratio, with birds in the high dose group having higher H/L ratios compared to controls

($P = 0.04$) (Figure 3-11). Time of exposure did not have a significant effect on the H/L ratios in ducks (Figure 3-12).

Descriptive statistics for the relative spleen weight/body weight ratios in 21-day-old chickens are summarized in Table 3-13. Simple comparisons indicate only slight differences among treatment groups and exposure times. A summary of the univariate comparisons between the fixed effect factors, herbicide treatment and time of exposure, and the mean spleen/body weight ratios is presented in Table 3-14. Exposure time and herbicide treatment were not considered to be important factors in the initial analysis ($P > 0.25$), and did not influence any differences between groups in the final univariate comparison model. Graphs showing the association between relative spleen weight and both fixed factors are presented in Figures 3-13 and 3-14. A summary of the descriptive statistics for the relative bursa of Fabricius weight/body weight ratios are presented in Table 3-15. Herbicide treatment and time of exposure were evaluated as potential fixed effect factors in a univariate comparison analysis model (Table 3-16). Only herbicide treatment was found to be a significant factor in the final model ($P = 0.0006$). Mean relative bursa weight was significantly reduced ($P = 0.04$) in birds from the low dose herbicide treatment group compared to control groups (Figure 3-15). The difference between control and high dose birds approached significance ($P = 0.08$) and high dose birds exhibited increased bursal weights. A compensatory immune response could be the explanation for lower bursa weights in the low dose group and higher bursa weights in the high dose group, but this effect is not certain. Relative bursa weights were also different between low dose and high dose herbicide spray treatments ($P = 0.0002$). There was no association between exposure times and mean relative bursa weights (Figure 3-16).

A descriptive summary of the relative spleen weights ($\log_{10}$ transformed to attain normality) of 21-day-old ducks is presented in Table 3-17. Although time of exposure approached significance in the final statistical model ($P = 0.07$), both fixed effect factors demonstrated no effect among treatment groups or exposure times (Table 3-18). Graphs showing the association between mean relative spleen weights and the type of spray treatment and time of exposure are presented in Figures 3-17 and 3-18, respectively. Descriptive statistics for relative bursa of Fabricius weights in 21-day-old ducklings are summarized in Table 3-19. Values were $\log_{10}$ transformed in order to attain normality. Both

Times of spray exposure were significantly different (P = 0.04) in the final comparison model. Relative bursa weights in the birds exposed to spray at the earlier stage of incubation (day 6) were significantly higher than those of birds treated at the later timepoint (day 21) (Table 3-20). However, the association between times of spray exposure and bursal weight are not a result of 2,4-D exposure, as there was no treatment effect in the final model. Graphs showing the relationship between mean bursa weight/body weight ratios and herbicide treatments and times of exposure are presented in Figures 3-19 and 3-20, respectively.

Histopathological examination of lymphoid organs (cross-sections of thymus, spleen and bursa of Fabricius) from birds treated *in ovo* with high dose 2,4-D spray and water (control) was performed by a wildlife pathologist. All three tissues were examined for overt pathology, particularly lymphocyte depletion or lymphoid atrophy. There was no evidence of herbicide-induced pathology, and no indication that the structure of lymphoid organs from 21-day-old chickens or ducks from the high dose groups was different from control birds.

Table 3-1. Descriptive summaries of serum dilutions (reciprocal values, $\log_{10}$ transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA. Serum was collected from 14- (a) and 21-day-old chicks (b) exposed *in ovo* to 2,4-D formulation spray and immunized with BSA at 7 and 14 days of age, to evaluate the effects of exposure on the primary and secondary antibody response, respectively.

(a) **Serum from 14-day-old chicks**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	27	2.747	0.429	0.083	2.602	2.602	2.903
	Late[2]	30	2.672	0.393	0.072	2.602	2.301	2.903
1 X 2,4-D (Low Dose)	Early[1]	31	2.719	0.483	0.087	2.602	2.301	3.204
	Late[2]	34	2.611	0.435	0.075	2.602	2.301	2.903
10 X 2,4-D (High Dose)	Early[1]	34	2.637	0.120	0.059	2.602	2.301	2.903
	Late[2]	34	2.850	0.537	0.092	2.602	2.602	3.204

(b) **Serum from 21-day-old chicks**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	27	3.171	0.467	0.090	3.204	2.903	3.505
	Late[2]	30	3.214	0.509	0.093	3.204	2.903	3.505
1 X 2,4-D (Low Dose)	Early[1]	31	3.097	0.463	0.083	3.204	2.903	3.505
	Late[2]	34	3.151	0.435	0.075	3.204	2.903	3.505
10 X 2,4-D (High Dose)	Early[1]	34	3.222	0.148	0.066	3.204	2.903	3.505
	Late[2]	34	3.346	0.458	0.079	3.555	2.903	3.806

[1] Day 6 and [2] Day 15 of incubation

Table 3-2. Summary of univariate comparisons between the fixed effect factors, herbicide treatment and time of exposure, and serum dilutions (reciprocal values, $\log_{10}$ transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA. Statistical comparisons are summarized for serum samples collected from 14- (a), and 21-day-old chicks (b) exposed *in ovo* to 2,4-D, and immunized with BSA at 7 and 14 days of age.

(a) **Serum from 14-day-old chicks**	**Regression coefficient (β)**	**95% Confidence Intervals for β**		***P* value**
		Lower	**Upper**	
Herbicide Treatment				
- High Dose[1]	0.036	-0.128	0.199	0.67
- Low[2]	-0.042	-0.207	0.123	0.62
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.016	-0.148	0.117	0.82
- Late[5]	Reference	-	-	-
(b) **Serum from 21-day-old chicks**				
Herbicide Treatment				
- High Dose[1]	0.090	-0.066	0.245	0.26
- Low Dose[2]	-0.066	-0.223	0.092	0.41
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.074	-0.210	0.061	0.28
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

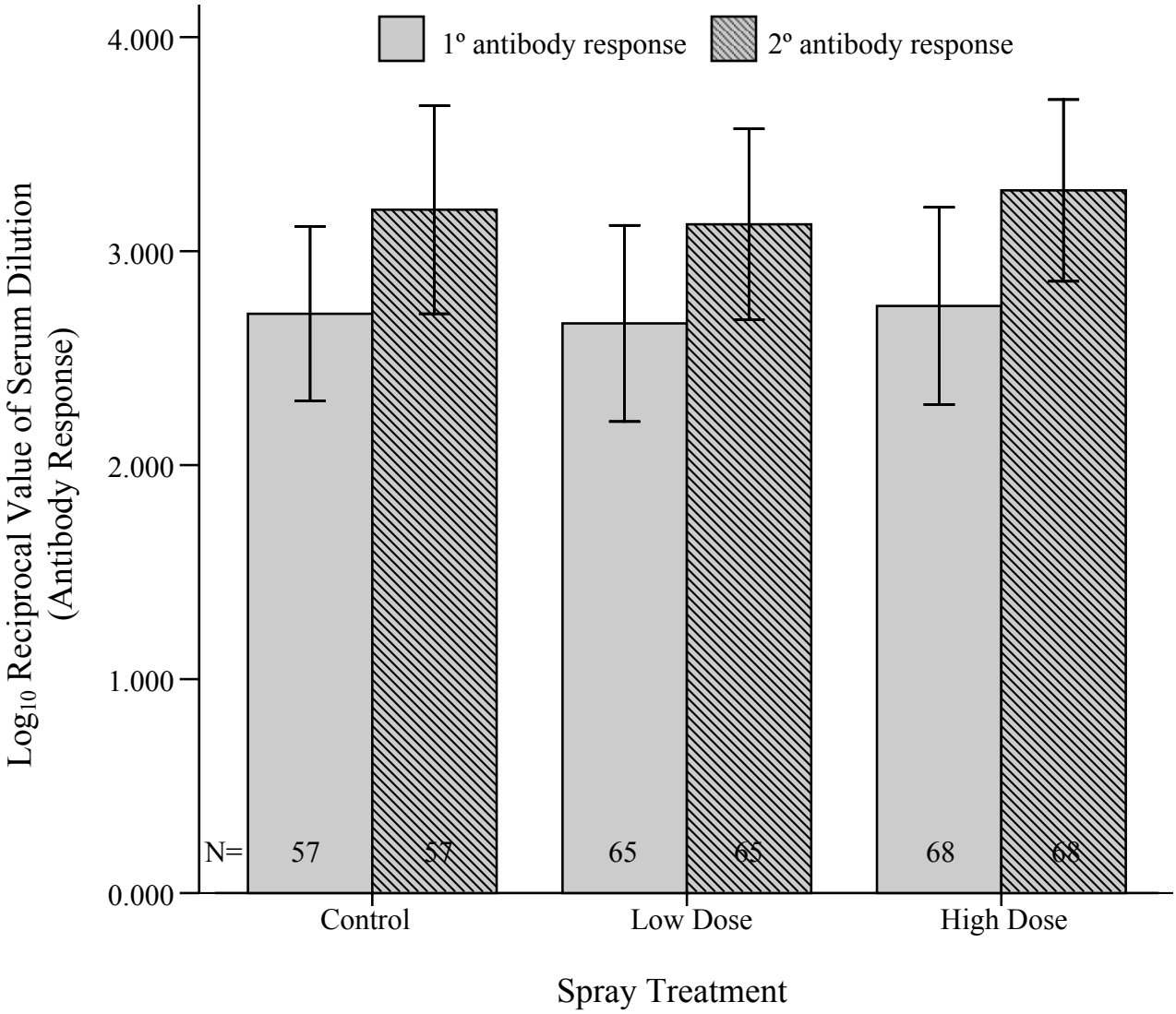

Figure 3-1. The simple association between the reciprocal of the highest dilution of serum (log transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA and *in ovo* 2,4-D herbicide exposure in chickens. The bars represent the mean reciprocal values of serum dilutions for samples collected from 14- and 21-day-old birds, and therefore reflect the strength of the primary and secondary humoral response, respectively. Bars are grouped into the following treatments: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean.

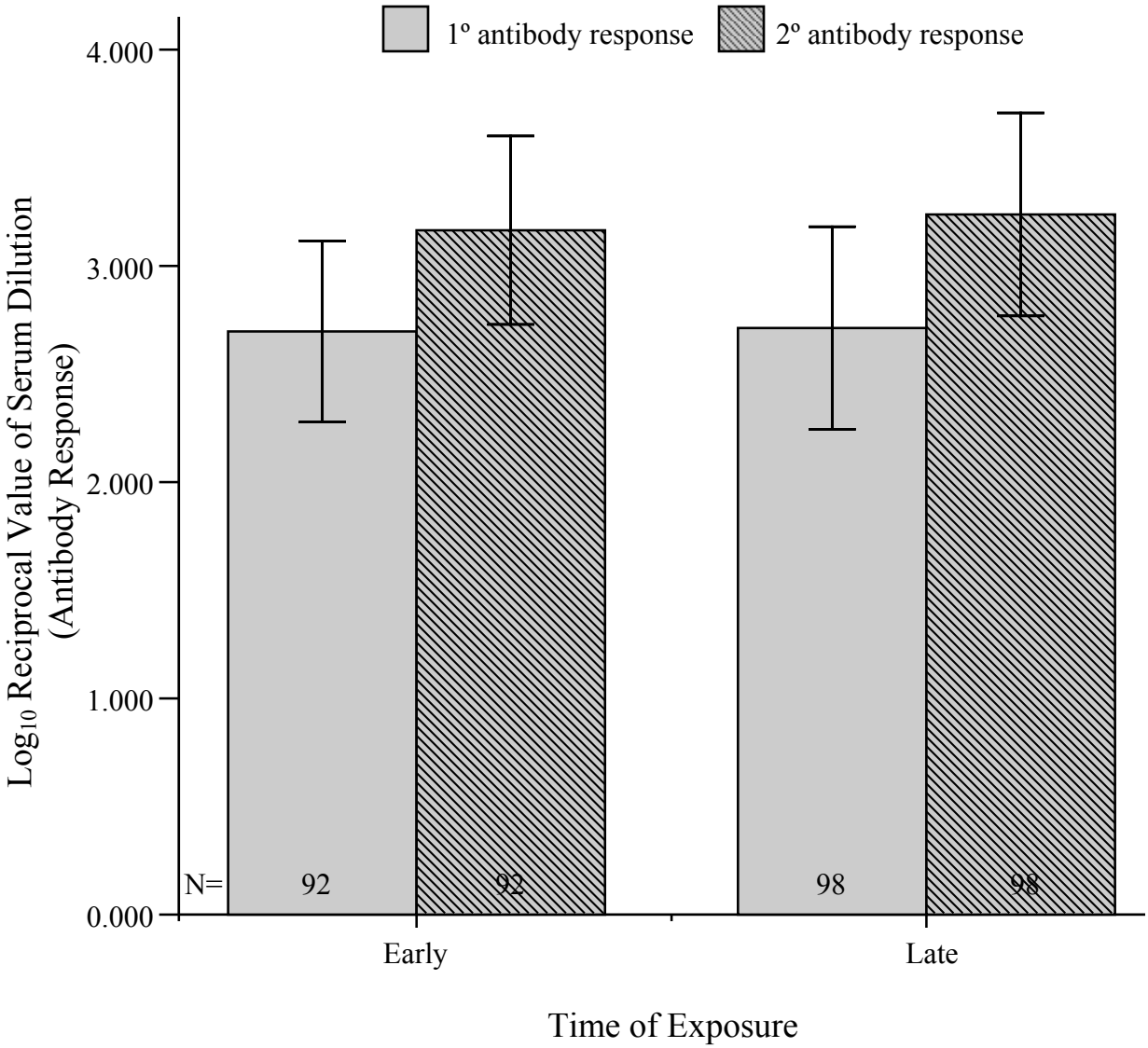

Figure 3-2. The simple association between the reciprocal of the highest dilution of serum (log transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA and the time of *in ovo* 2,4-D herbicide spray application in chickens. The bars represent the mean reciprocal values of serum dilutions for samples collected from 14 and 21-day-old birds, and therefore reflect the strength of the primary and secondary humoral response, respectively. Bars are grouped into the following exposure groups: early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

Table 3-3. Descriptive summaries of the serum dilutions (reciprocal values, log transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA. Serum was collected from 14- (a) and 21-day-old ducks (b) exposed *in ovo* to 2,4-D formulation spray, and immunized with BSA at 7 and 14 days of age, to evaluate the effects of exposure on the primary and secondary antibody response, respectively.

(a) **Serum from 14-day-old ducks**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	18	0.669	0.407	0.096	0.602	0.527	0.978
	Late[2]	18	0.485	0.463	0.109	0.452	0.000	0.677
1 X 2,4-D (Low Dose)	Early[1]	9	0.602	0.563	0.188	0.602	0.151	0.903
	Late[2]	7	0.946	0.613	0.232	0.903	0.602	1.505
10 X 2,4-D (High Dose)	Early[1]	10	0.482	0.406	0.129	0.301	0.301	0.753
	Late[2]	22	0.698	0.612	0.130	0.602	0.226	1.279

(b) **Serum from 21-day-old ducks**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	18	1.204	0.526	0.124	1.204	0.602	1.806
	Late[2]	18	1.171	0.325	0.077	1.204	0.903	1.505
1 X 2,4-D (Low Dose)	Early[1]	9	1.003	0.499	0.166	0.903	0.602	1.505
	Late[2]	7	1.118	0.513	0.194	0.903	0.602	1.806
10 X 2,4-D (High Dose)	Early[1]	10	1.294	0.619	0.196	1.505	0.828	1.806
	Late[2]	22	1.108	0.459	0.098	1.204	0.903	1.505

[1] Day 6 and [2] Day 21 of incubation

Table 3-4. Summary of univariate comparisons between the fixed effect factors, herbicide treatment and time of exposure, and serum dilutions (reciprocal values) that demonstrated a positive reaction against BSA in the ELISA. Statistical comparisons are summarized for serum samples collected from (a) 14- and (b) 21-day-old ducklings exposed *in ovo* to 2,4-D, and immunized with BSA at 7 and 14 days of age.

(a) **Serum from 14-day-old ducks**	**Regression coefficient (β)**	**95% Confidence Intervals for β**		***P* value**
		Lower	**Upper**	
Herbicide Treatment				
- High Dose[1]	0.082	-0.133	0.297	0.45
- Low[2]	0.168	-0.099	0.434	0.21
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.123	-0.327	0.082	0.24
- Late[5]	Reference	-	-	-
(b) **Serum from 21-day-old ducks**				
Herbicide Treatment				
- High Dose[1]	-0.043	-0.256	0.171	0.69
- Low Dose[2]	-0.186	-0.450	0.079	0.17
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.015	-0.219	0.189	0.88
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 21 of incubation

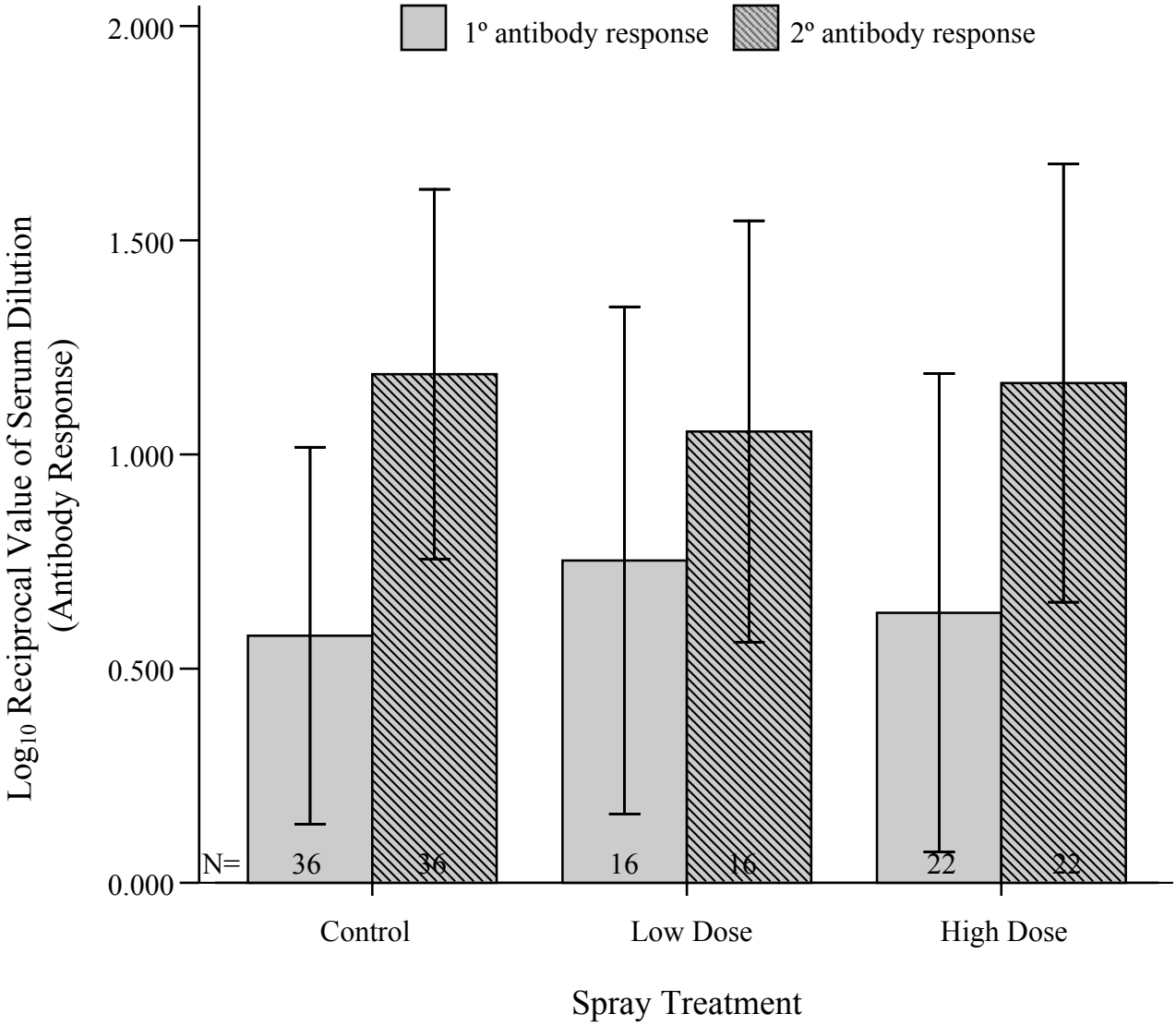

Figure 3-3. The simple association between the reciprocal of the highest dilution of serum (log transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA and *in ovo* 2,4-D herbicide exposure in ducks. The bars represent the mean reciprocal values of serum dilutions for samples collected from 14- and 21-day-old birds, and therefore reflect the strength of the primary and secondary humoral response, respectively. Bars are grouped into the following treatments: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean.

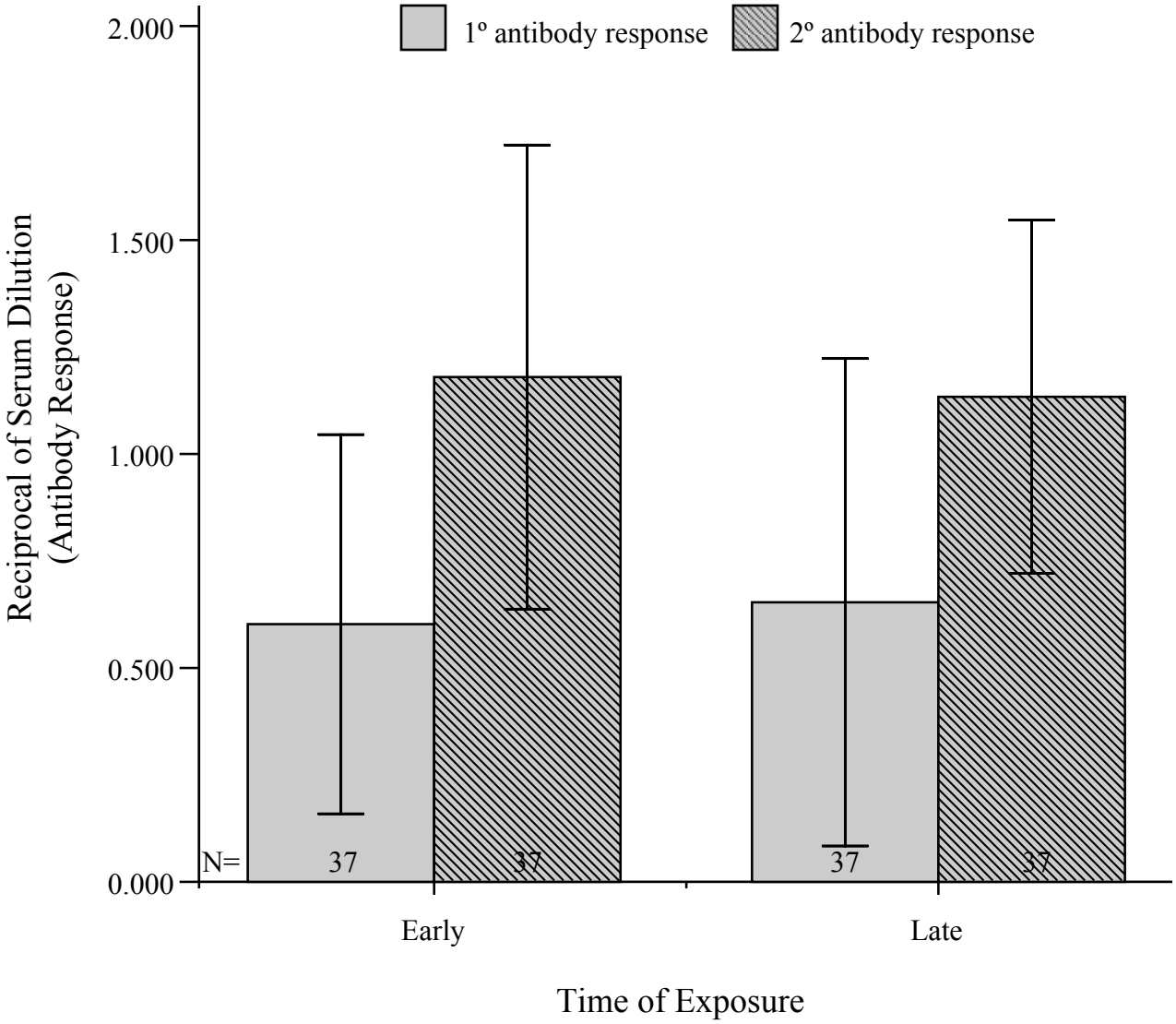

Figure 3-4. The simple association between the reciprocal of the highest dilution of serum (log transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA and the time of *in ovo* 2,4-D herbicide spray application in ducks. The bars represent the mean reciprocal values of serum dilutions for samples collected from 14- and 21-day-old birds, and therefore reflect the strength of the primary and secondary humoral response, respectively. Bars are grouped into the following exposure groups: early (day 6 of incubation) and late (day 21 of incubation). Error bars represent ± SD of the mean.

Table 3-5. Descriptive summary of the DTH response in 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray. DTH response was assessed by measuring differences in wing web thickness following intradermal BSA injection. All mean values of the DTH outcome represent a positive change (increase) in wing web thickness (mm).

							Percentiles	
Treatment	**Exposure Time**	**N**	**Mean**	**SD**	**SE**	**Median**	**25th**	**75th**
Water (Negative Control)	Early[1]	13	0.13	0.11	0.03	0.10	0.06	0.24
	Late[2]	15	0.13	0.12	0.03	0.12	0.06	0.21
1 X 2,4-D (Low Dose)	Early[1]	15	0.10	0.08	0.02	0.08	0.02	0.17
	Late[2]	13	0.10	0.08	0.02	0.12	0.03	0.19
10 X 2,4-D (High Dose)	Early[1]	15	0.15	0.08	0.02	0.13	0.09	0.23
	Late[2]	14	0.13	0.08	0.02	0.13	0.07	0.20

[1] Day 6 and [2] Day 15 of incubation

Table 3-6. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the wing web DTH response to BSA injection, measured in 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	**95% Confidence Intervals for β**		***P* value**
		Lower	**Upper**	
Herbicide Treatment				
- High Dose[1]	0.01	-0.03	0.05	0.71
- Low Dose[2]	-0.03	-0.07	0.02	0.21
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.01	-0.03	0.04	0.78
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

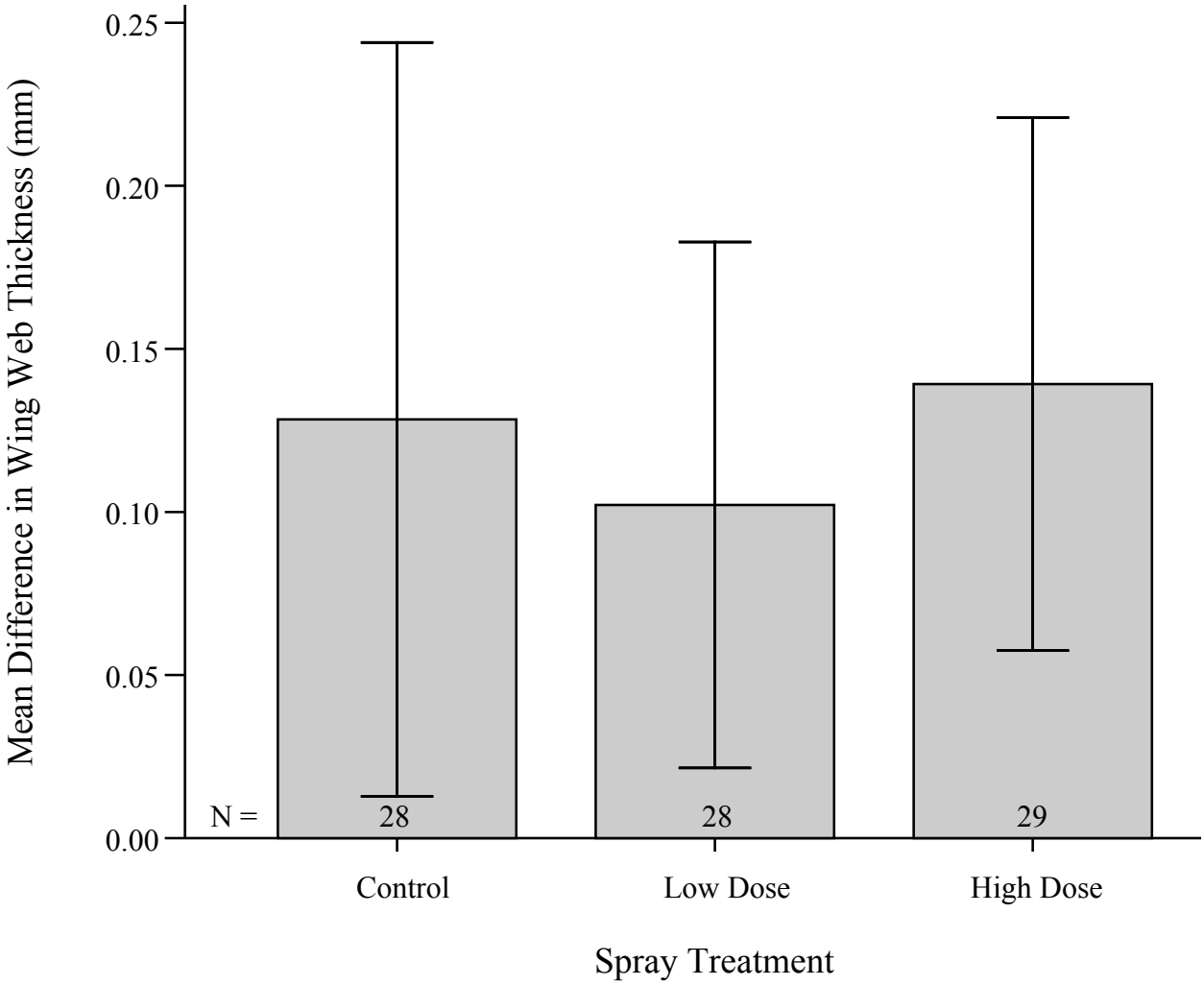

Figure 3-5. The simple association between DTH response (increase in wing web thickness, mm) in 21-day-old domestic chickens and *in ovo* 2,4-D herbicide exposure. The bars represent mean DTH response for the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean.

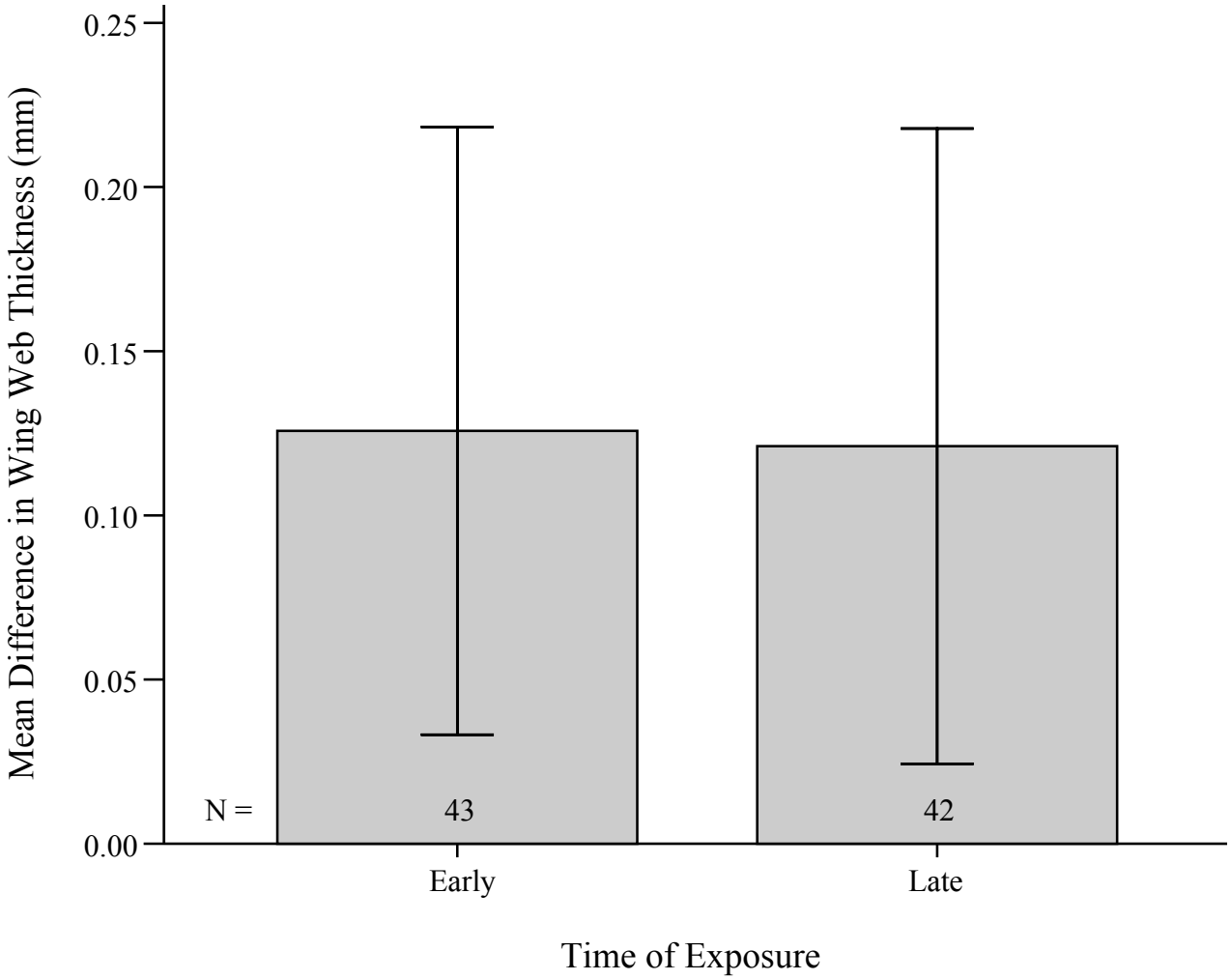

Figure 3-6. The simple association between DTH response (increase in wing web thickness, mm) in 21-day-old domestic chickens and the time of *in ovo* 2,4-D herbicide spray application. The bars represent mean DTH response for the following exposure groups: early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

Table 3-7. Descriptive summary of the DTH response in 21-day-old domestic ducks exposed *in ovo* to 2,4-D formulation spray. DTH response was assessed by measuring differences in wing web thickness following intradermal BSA injection. All mean values of the DTH outcome represent a positive change (increase) in wing web thickness (mm).

							Percentiles	
Treatment	**Exposure Time**	**N**	**Mean**	**SD**	**SE**	**Median**	**25th**	**75th**
Water (Negative Control)	Early[1]	19	0.24	0.15	0.03	0.26	0.14	0.37
	Late[2]	18	0.20	0.16	0.04	0.18	0.10	0.27
1 X 2,4-D (Low Dose)	Early[1]	9	0.13	0.16	0.05	0.08	0.01	0.28
	Late[2]	7	0.18	0.13	0.05	0.21	0.08	0.31
10 X 2,4-D (High Dose)	Early[1]	10	0.25	0.16	0.05	0.19	0.13	0.35
	Late[2]	22	0.21	0.11	0.02	0.21	0.14	0.28

[1] Day 6 and [2] Day 21 of incubation

Table 3-8. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the wing web DTH response to BSA injection, measured in 21-day-old domestic ducks exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	**95% Confidence Intervals for β**		***P* value**
		Lower	**Upper**	
Herbicide Treatment				
- High Dose[1]	0.01	-0.06	0.08	0.85
- Low Dose[2]	-0.06	-0.15	0.03	0.16
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.02	-0.04	0.09	0.48
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 21 of incubation

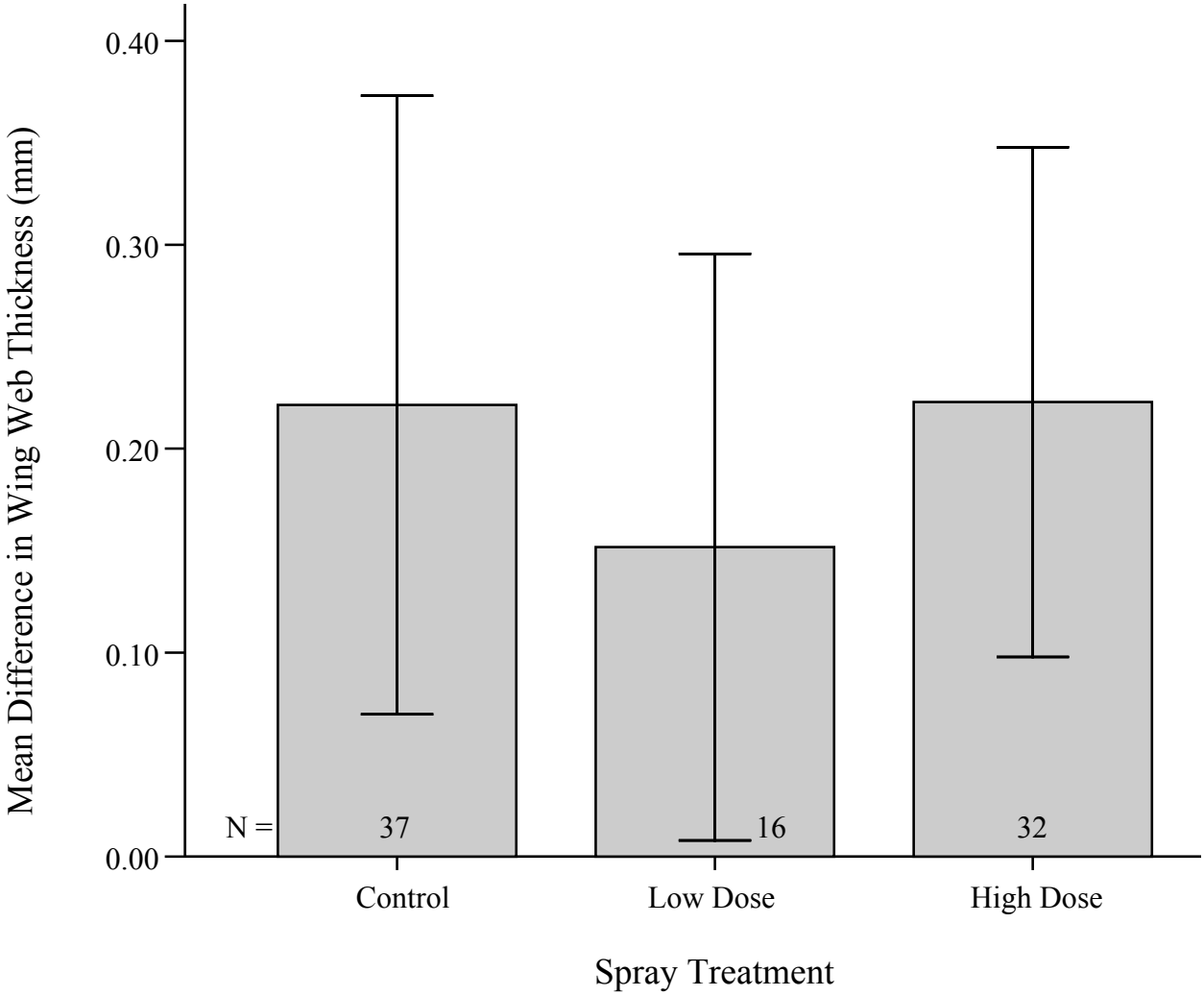

Figure 3-7. The simple association between DTH response (increase in wing web thickness, mm) in 21-day-old domestic ducks and *in ovo* 2,4-D herbicide exposure. The bars represent mean DTH response for the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean.

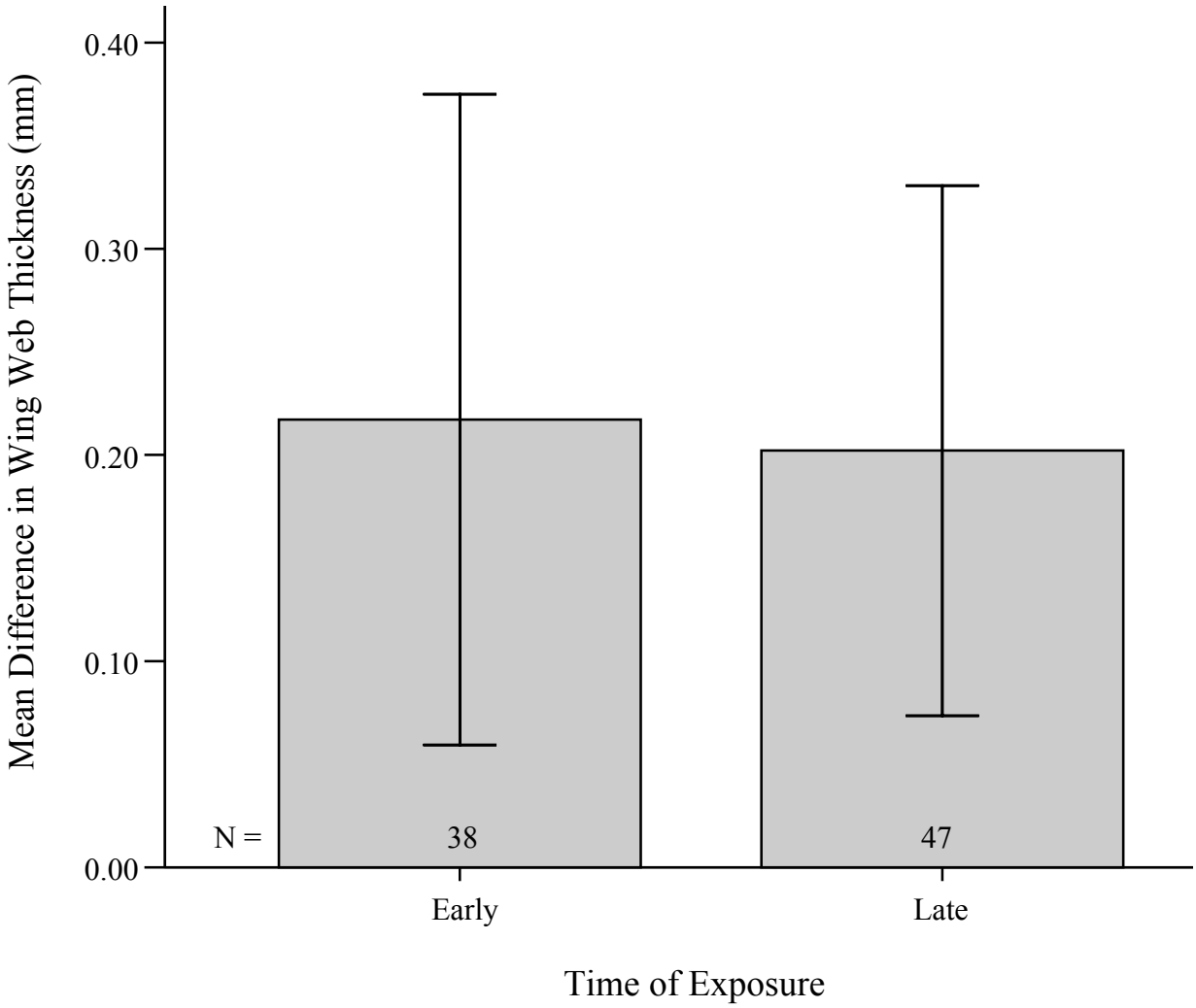

Figure 3-8. The simple association between DTH response (increase in wing web thickness, mm) in 21-day-old domestic ducks and the time of *in ovo* 2,4-D herbicide spray application. The bars represent mean DTH response for the following exposure groups: early (day 6 of incubation) and late (day 21 of incubation). Error bars represent ± SD of the mean.

Table 3-9. Descriptive summary of the H/L ratios in peripheral blood from 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	33	0.540	0.172	0.030	0.533	0.444	0.627
	Late[2]	33	0.499	0.190	0.033	0.460	0.353	0.575
1 X 2,4-D (Low Dose)	Early[1]	31	0.491	0.234	0.042	0.435	0.294	0.679
	Late[2]	34	0.542	0.247	0.042	0.480	0.354	0.717
10 X 2,4-D (High Dose)	Early[1]	34	0.479	0.173	0.030	0.496	0.351	0.575
	Late[2]	34	0.544	0.177	0.030	0.529	0.432	0.667

[1] Day 6 and [2] Day 15 of incubation

Table 3-10. Summary of univariate comparisons between the fixed effect factors, time of exposure and herbicide treatment, and the H/L ratios in peripheral blood from 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	-0.022	-0.192	0.149	0.80
- Low Dose[2]	-0.051	-0.223	0.121	0.56
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.066	-0.198	0.066	0.32
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

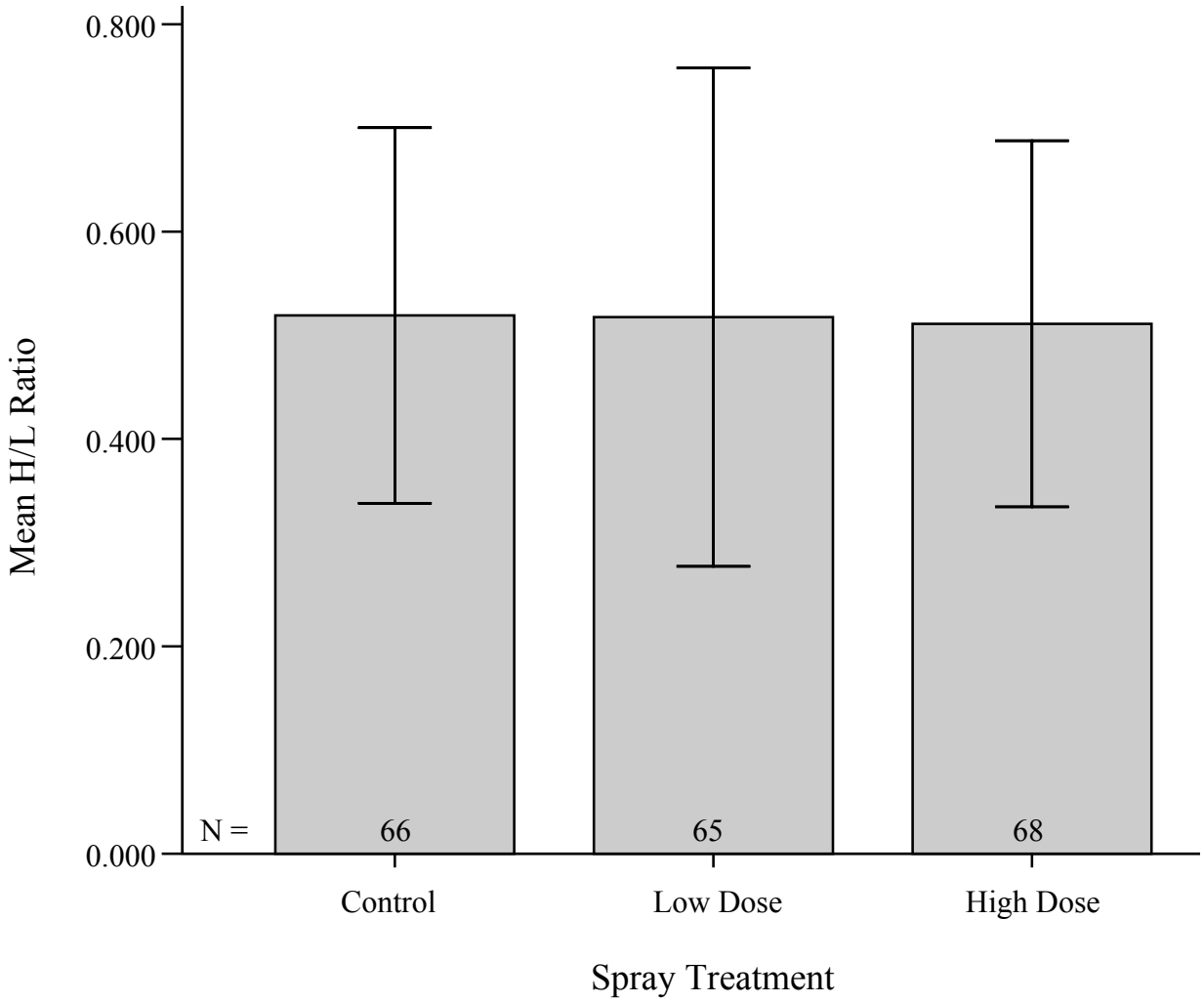

Figure 3-9. The simple association between heterophil/lymphocyte (H/L) ratio in peripheral blood from 21-day-old domestic chickens and *in ovo* 2,4-D commercial herbicide exposure. The bars represent mean H/L ratios for the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean.

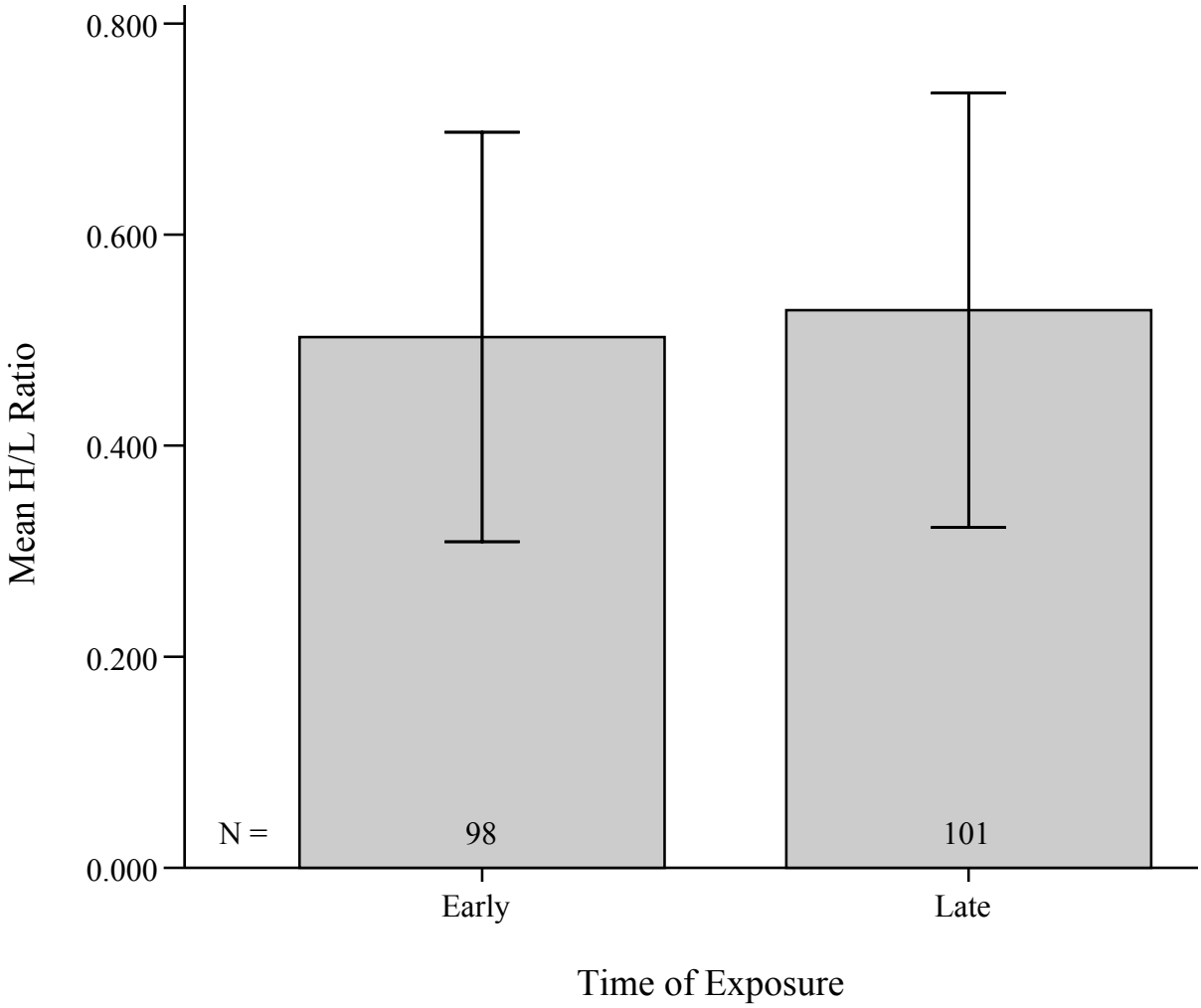

Figure 3-10. The simple association between heterophil/lymphocyte (H/L) ratio in peripheral blood from 21-day-old domestic chickens and the time of *in ovo* 2,4-D herbicide spray application. The bars represent mean H/L ratios for the following exposure groups: early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

Table 3-11. Descriptive summary of the H/L ratios in peripheral blood from 21-day-old domestic ducks exposed *in ovo* to a 2,4-D formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	19	0.800	0.303	0.069	0.700	0.549	1.000
	Late[2]	18	0.595	0.304	0.072	0.632	0.390	0.821
1 X 2,4-D (Low Dose)	Early[1]	9	0.783	0.470	0.157	0.706	0.421	1.075
	Late[2]	7	0.877	0.117	0.044	0.889	0.787	0.977
10 X 2,4-D (High Dose)	Early[1]	10	0.931	0.484	0.153	0.851	0.681	1.058
	Late[2]	22	0.869	0.364	0.078	0.853	0.618	1.081

[1] Day 6 and [2] Day 21 of incubation

Table 3-12. Summary of univariate comparisons between the fixed effect factors, time of exposure and herbicide treatment, and the H/L ratios in peripheral blood from 21-day-old domestic ducks exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	0.186	0.009	0.363	0.04*
- Low Dose[2]	0.126	-0.092	0.344	0.25
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.063	-0.115	0.242	0.48
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 21 of incubation

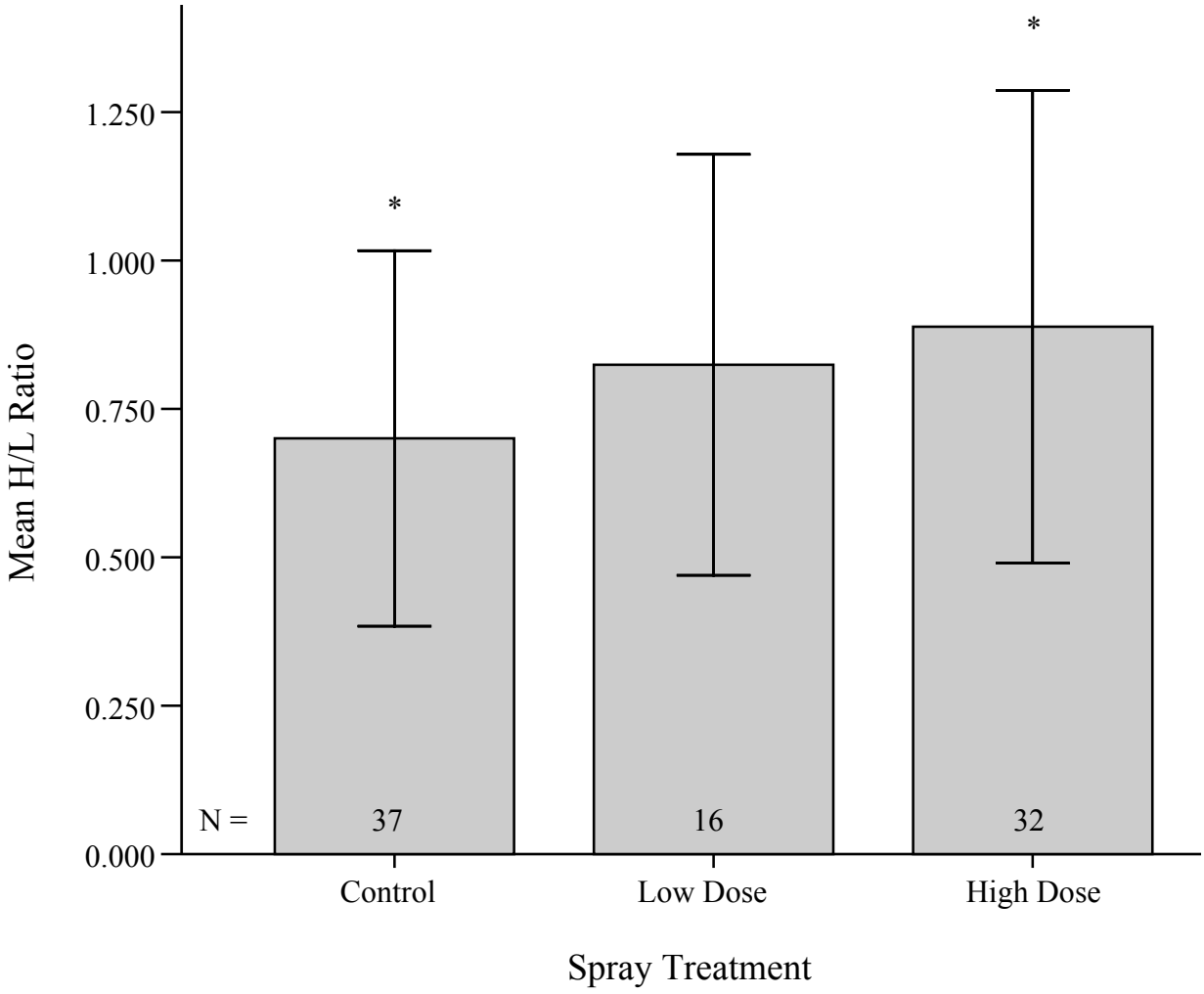

Figure 3-11. The simple association between heterophil/lymphocyte (H/L) ratio in peripheral blood from 21-day-old domestic ducks and *in ovo* 2,4-D herbicide exposure. The bars represent mean H/L ratios for the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean. *Mean H/L ratio was significantly different between water control and high dose herbicide spray treatments ($P = 0.0395$).

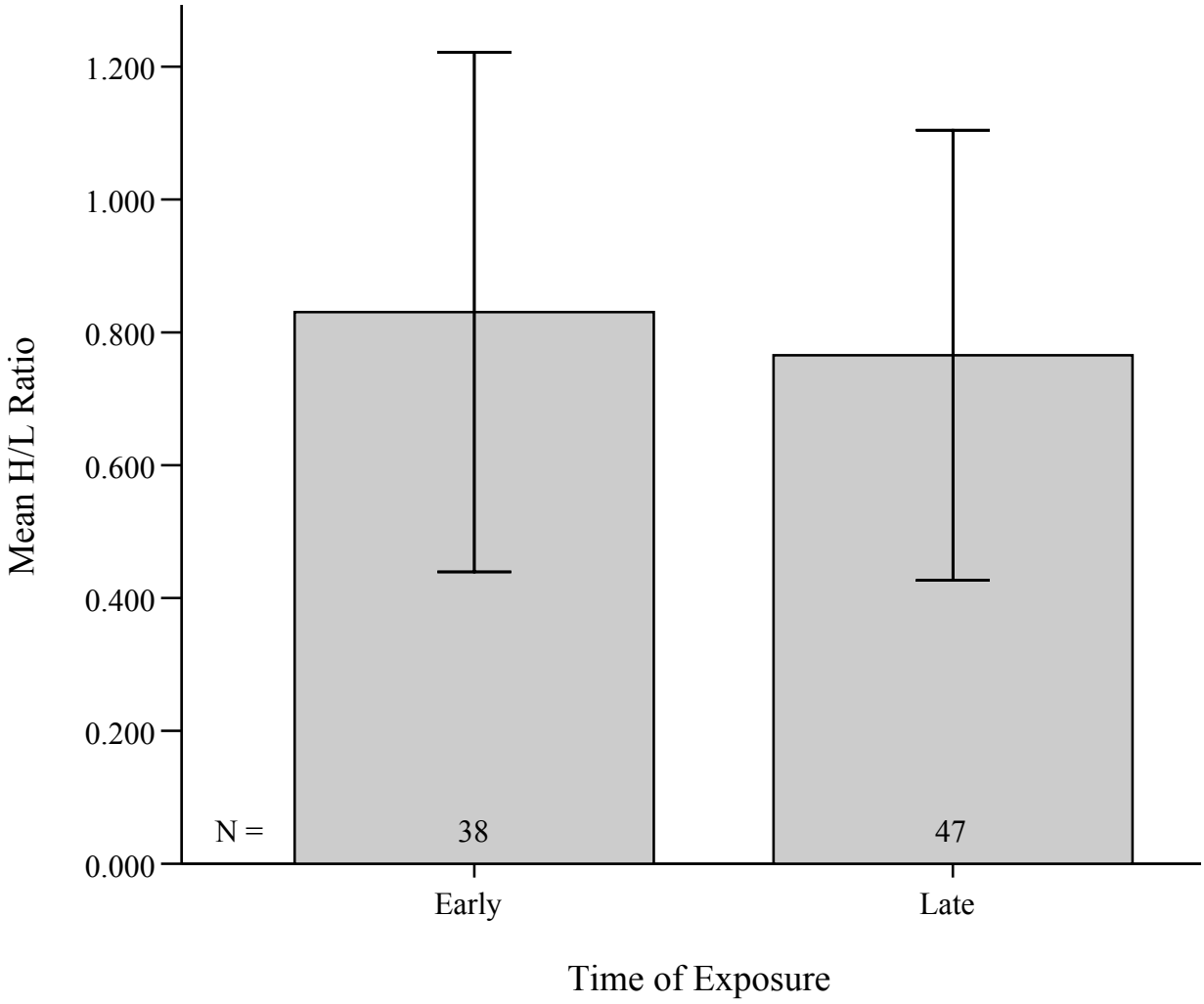

Figure 3-12. The simple association between heterophil/lymphocyte (H/L) ratios in peripheral blood from 21-day-old domestic ducks and the time of *in ovo* 2,4-D herbicide spray application. The bars represent mean H/L ratios for the following exposure groups: early (day 6 of incubation) and late (day 21 of incubation). Error bars represent ± SD of the mean.

Table 3-13. Descriptive summary of the relative spleen/body weight ratio measured in 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	19	0.134	0.026	0.006	0.140	0.120	0.150
	Late[2]	20	0.165	0.039	0.009	0.170	0.133	0.190
1 X 2,4-D (Low Dose)	Early[1]	25	0.148	0.033	0.007	0.150	0.130	0.170
	Late[2]	15	0.133	0.035	0.009	0.130	0.110	0.160
10 X 2,4-D (High Dose)	Early[1]	27	0.152	0.026	0.005	0.150	0.140	0.170
	Late[2]	23	0.156	0.039	0.008	0.150	0.120	0.180

[1] Day 6 and [2] Day 15 of incubation

Table 3-14. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the relative spleen/body weight ratio measured in 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	0.002	-0.014	0.018	0.79
- Low Dose[2]	-0.008	-0.025	0.009	0.33
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.008	-0.021	0.005	0.24
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

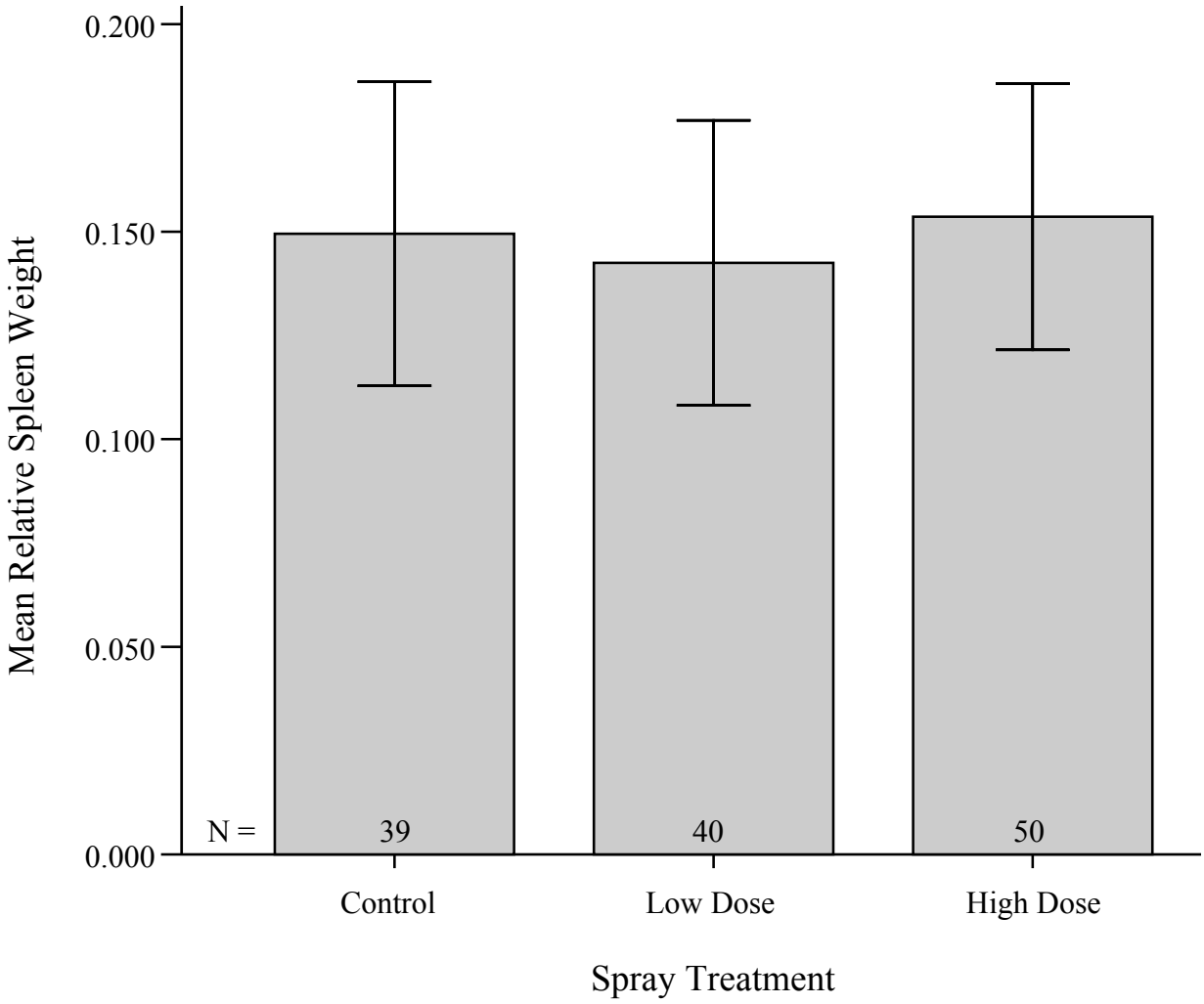

Figure 3-13. The simple association between mean spleen weight/body weight ratios in 21-day-old domestic chickens and *in ovo* 2,4-D herbicide exposure. The bars represent the mean relative spleen weights for the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean.

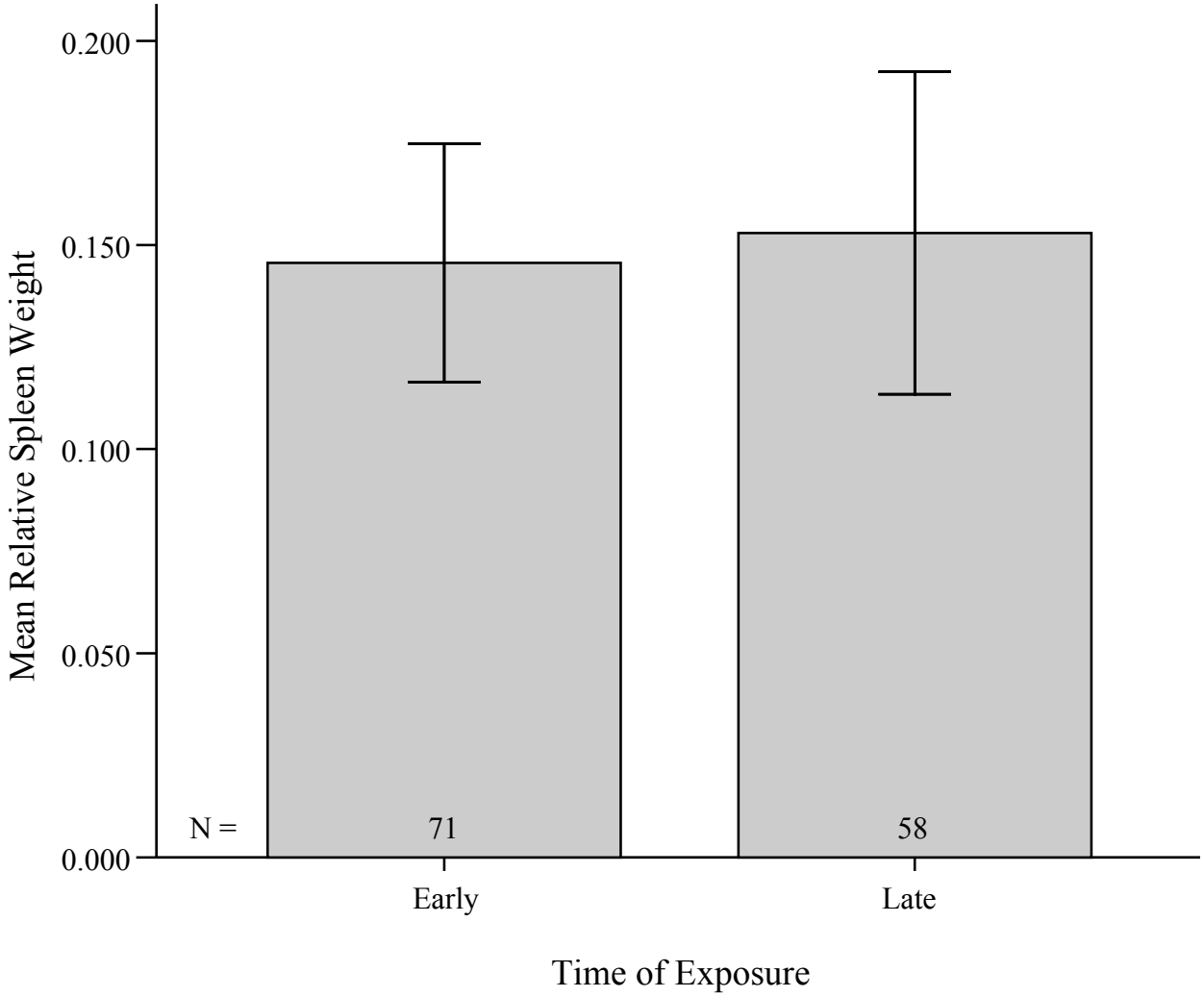

Figure 3-14. The simple association between mean spleen weight/body weight ratios in 21-day-old domestic chickens and the time of *in ovo* 2,4-D herbicide spray application. The bars represent the mean relative spleen weights for the following exposure groups; early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

Table 3-15. Descriptive summary of the relative bursa of Fabricius weight/body weight ratio measured in 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	19	0.440	0.085	0.020	0.470	0.390	0.510
	Late[2]	20	0.489	0.115	0.026	0.485	0.423	0.558
1 X 2,4-D (Low Dose)	Early[1]	25	0.445	0.114	0.023	0.450	0.360	0.535
	Late[2]	15	0.383	0.086	0.022	0.410	0.300	0.480
10 X 2,4-D (High Dose)	Early[1]	27	0.505	0.103	0.020	0.510	0.440	0.560
	Late[2]	23	0.519	0.123	0.026	0.490	0.460	0.560

[1] Day 6 and [2] Day 15 of incubation

Table 3-16. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the relative bursa of Fabricius weight/body weight ratio measured in 21-day-old domestic chickens exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	0.039	-0.005	0.083	0.08
- Low Dose[2]	-0.048	-0.095	-0.002	0.04*
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.002	-0.052	0.048	0.94
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

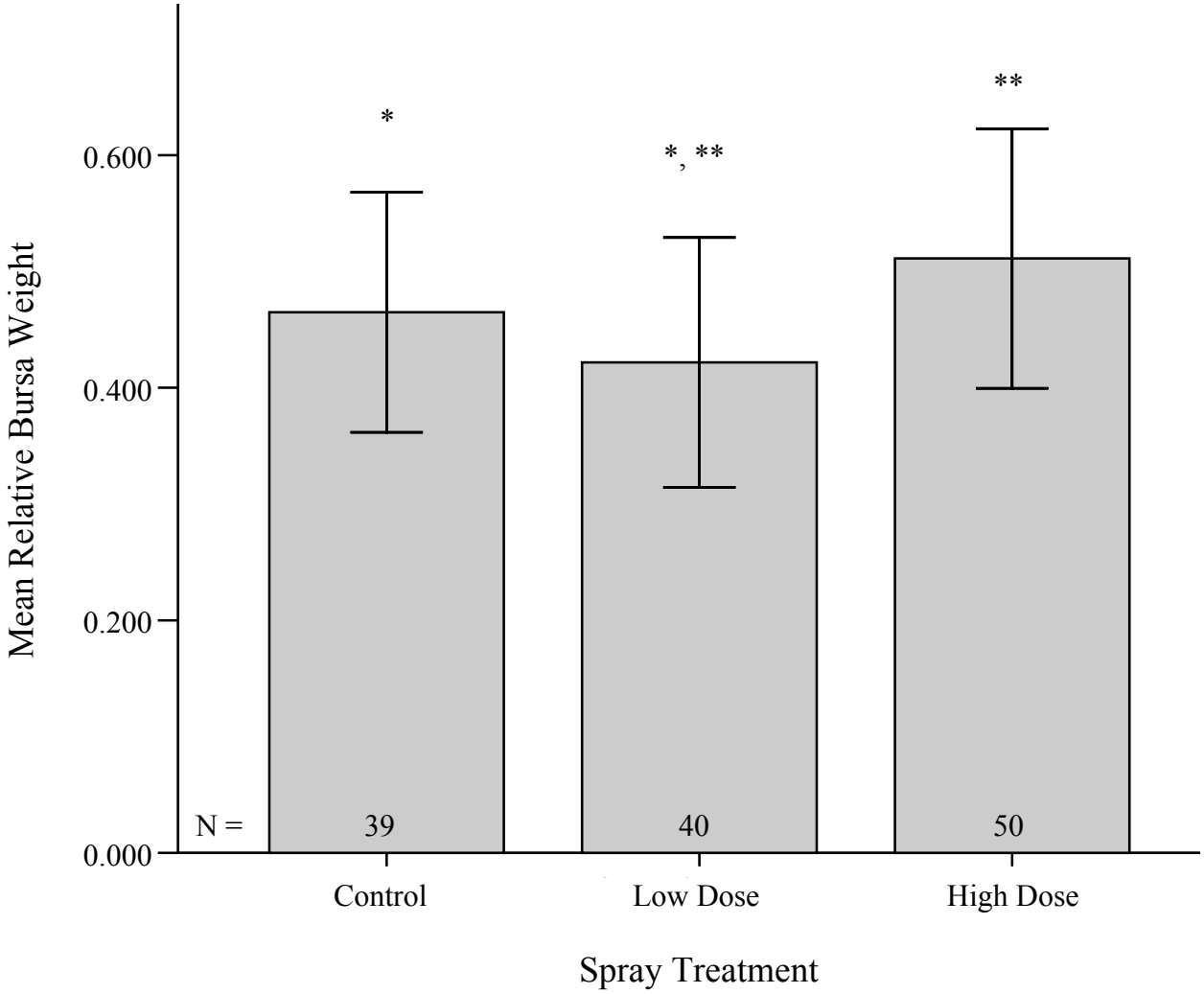

Figure 3-15. The simple association between mean bursa of Fabricius weight/body weight ratios in 21-day-old domestic chickens and *in ovo* 2,4-D herbicide exposure. The bars represent the mean relative bursa weights for the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean. *Mean bursa weights/body weights are significantly different between water control and low dose herbicide spray treatments (P = 0.0414). **Mean relative bursa weights are significantly different between the low and high dose herbicide groups (P = 0.0002).

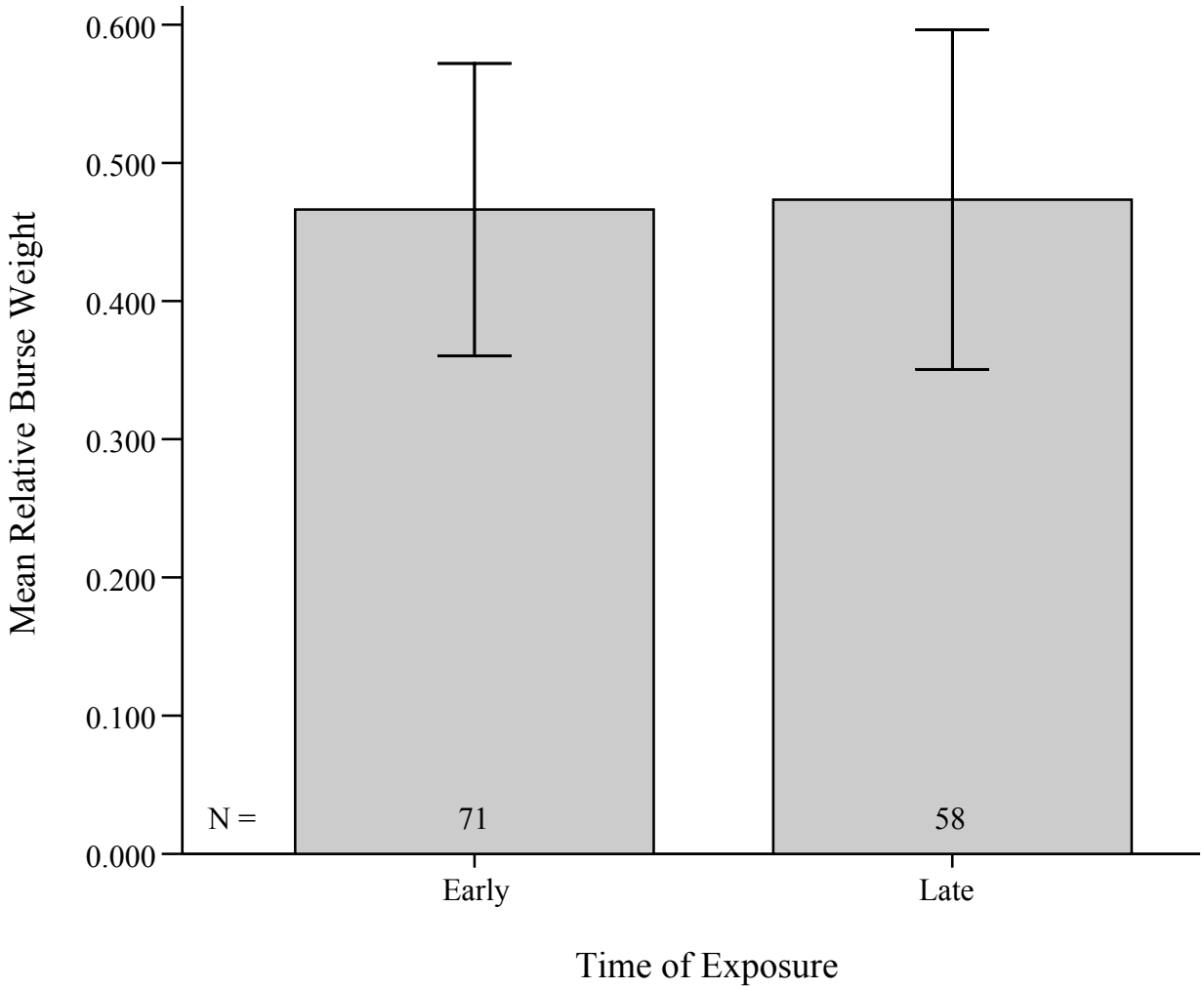

Figure 3-16. The simple association between mean bursa of Fabricius weight/body weight ratios in 21-day-old domestic chickens and the time of *in ovo* 2,4-D herbicide spray application. The bars represent the mean relative bursa weights for the following exposure groups: early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

Table 3-17. Descriptive summary of the relative spleen/body weight ratio (log_{10} transformed to attain normality) measured in 21-day-old domestic ducks exposed *in ovo* to 2,4-D formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	19	1.403	0.106	0.024	1.046	1.000	1.155
	Late[2]	18	0.978	0.064	0.015	0.959	0.949	1.000
1 X 2,4-D (Low Dose)	Early[1]	9	0.986	0.116	0.039	1.046	0.854	1.071
	Late[2]	7	0.933	0.110	0.042	0.921	0.854	1.046
10 X 2,4-D (High Dose)	Early[1]	10	1.007	0.122	0.039	1.046	0.878	1.073
	Late[2]	22	1.001	0.087	0.019	1.002	0.921	1.097

[1] Day 6 and [2] Day 21 of incubation

Table 3-18. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the relative spleen/body weight ratio (log_{10} transformed to attain normality) measured in 21-day-old domestic ducks exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	-0.006	-0.069	0.057	0.85
- Low Dose[2]	-0.060	-0.133	0.013	0.11
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.045	-0.033	0.094	0.07
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 21 of incubation

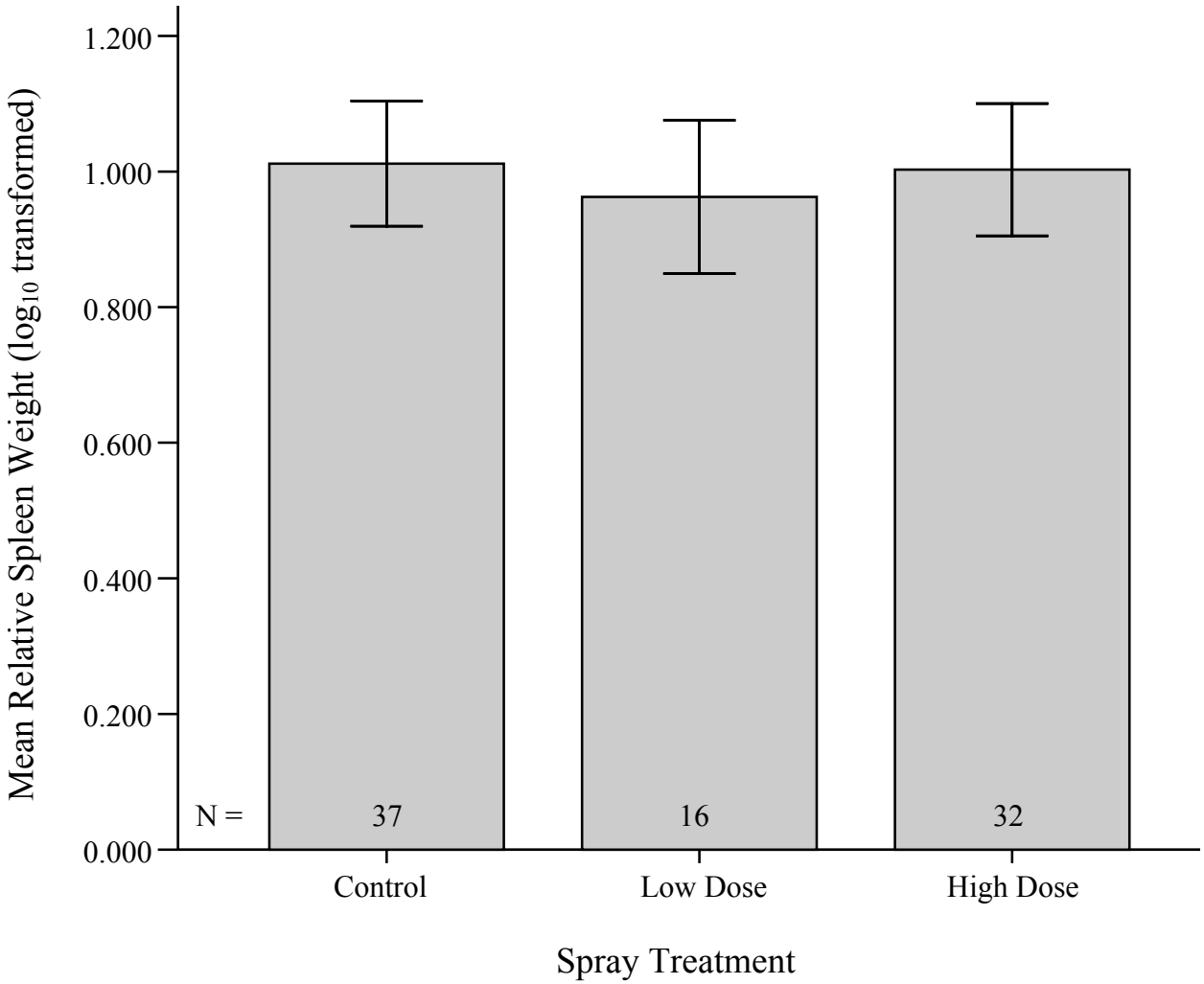

Figure 3-17. The simple association between mean spleen weight/body weight ratios in 21-day-old domestic ducks (log_{10} transformed to attain normality) and *in ovo* 2,4-D herbicide exposure. The bars represent the mean relative spleen weights for the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean.

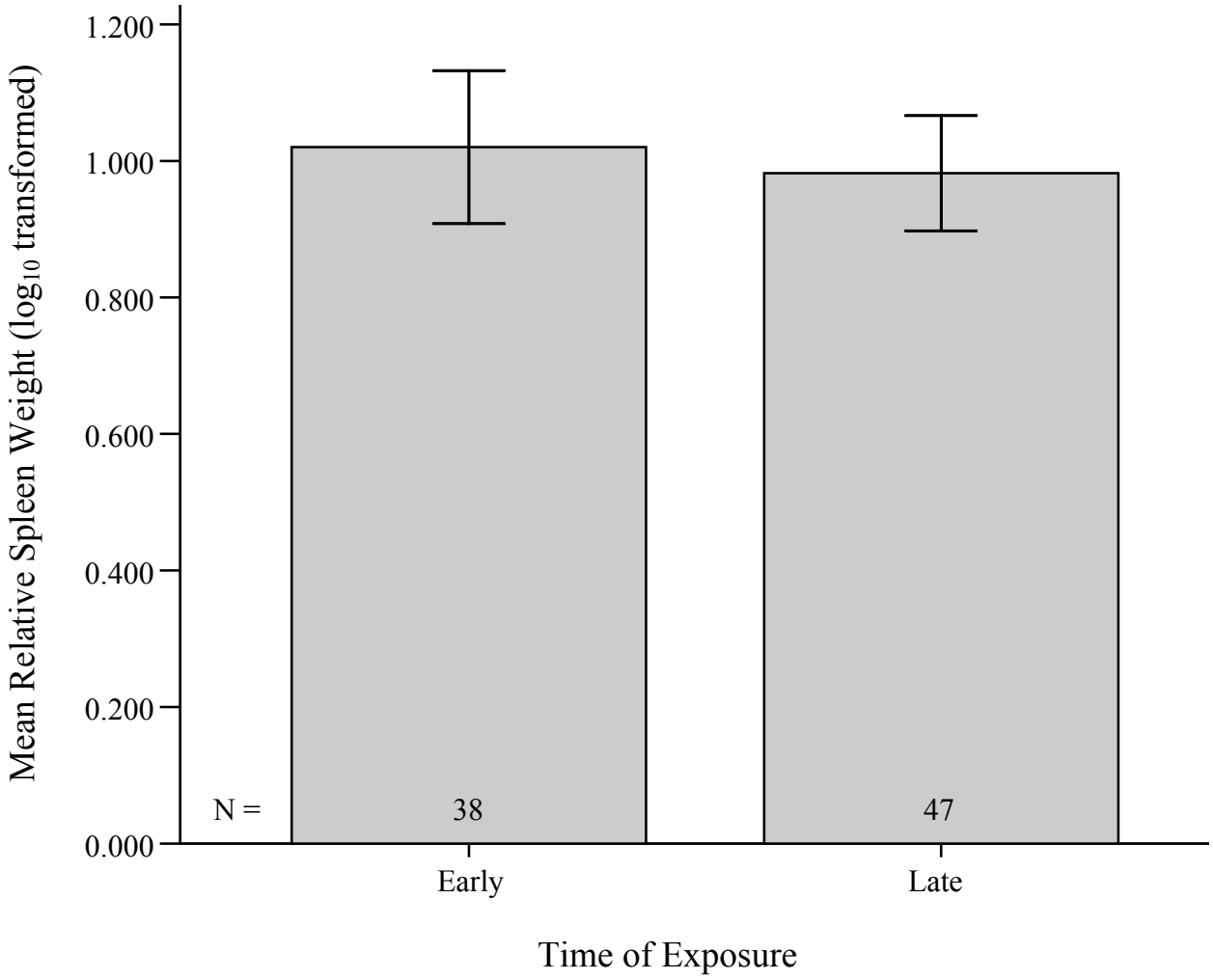

Figure 3-18. The simple association between mean spleen weight/body weight ratios (log_{10} transformed to attain normality) in 21-day-old domestic ducks and the time of *in ovo* 2,4-D herbicide spray application. The bars represent the mean relative spleen weights for the following exposure groups: early (day 6 of incubation) and late (day 21 of incubation). Error bars represent ± SD of the mean.

Table 3-19. Descriptive summary of the relative bursa of Fabricius weight/body weight ratio (log_{10} transformed to attain normality) measured in 21-day-old domestic ducks exposed *in ovo* to 2,4-D formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	19	0.930	0.129	0.029	0.959	0.886	1.000
	Late[2]	18	0.803	0.091	0.022	0.796	0.745	0.862
1 X 2,4-D (Low Dose)	Early[1]	9	0.862	0.063	0.021	0.886	0.812	0.903
	Late[2]	7	0.834	0.103	0.039	0.824	0.770	0.921
10 X 2,4-D (High Dose)	Early[1]	10	0.921	0.108	0.034	0.903	0.824	1.000
	Late[2]	22	0.853	0.091	0.019	0.854	0.796	0.895

[1] Day 6 and [2] Day 21 of incubation

Table 3-20. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the relative bursa of Fabricius weight/body weight ratio (log_{10} transformed to attain normality) measured in 21-day-old domestic ducks exposed *in ovo* to 2,4-D formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	0.023	-0.063	0.108	0.59
- Low Dose[2]	-0.018	-0.113	0.076	0.70
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.066	0.002	0.129	0.04*
- Late[5]	Reference	-	-	-

[1] 10 X 2,4-D commercial herbicide formulation spray
[2] 1 X 2,4-D commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 21 of incubation

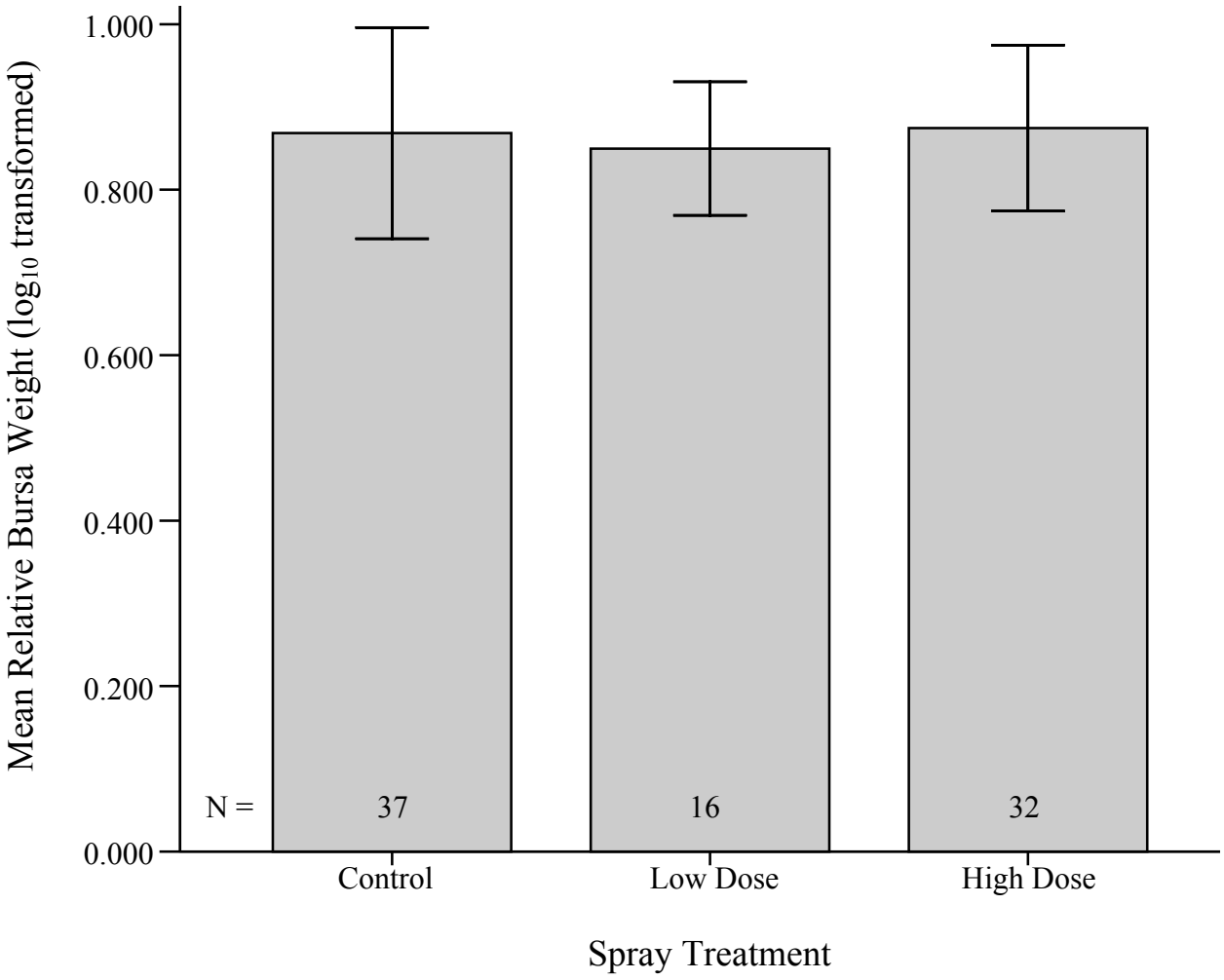

Figure 3-19. The simple association between mean bursa of Fabricius weight/body weight ratios (log_{10} transformed to attain normality) in 21-day-old domestic ducks and *in ovo* 2,4-D herbicide exposure. The bars represent the mean relative bursa weights for the following groups: control (water), low dose (1 X 2,4-D), and high dose (10 X 2,4-D). Error bars represent ± SD of the mean.

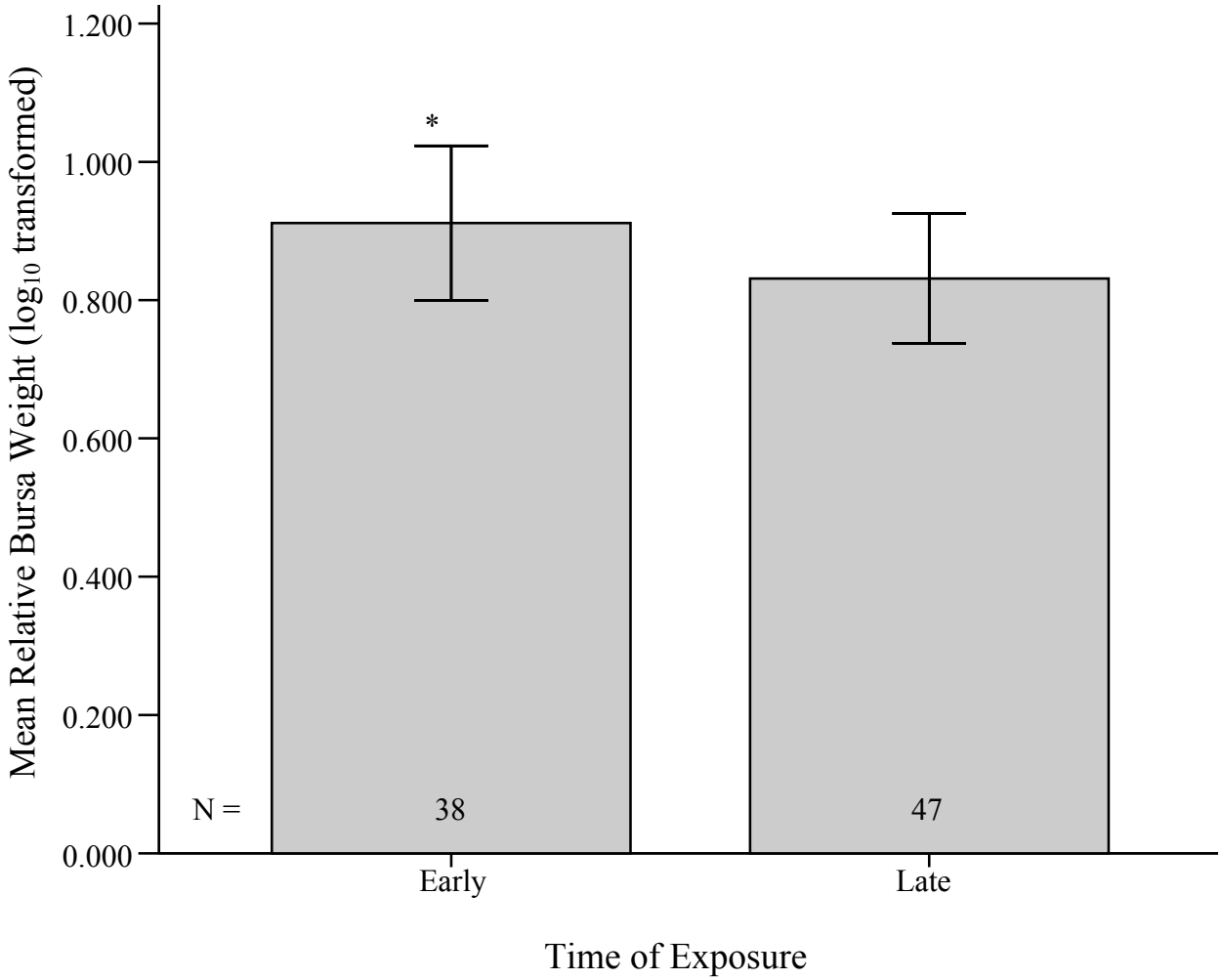

Figure 3-20. The simple association between mean bursa of Fabricius weight/body weight ratios (log_{10} transformed to attain normality) in 21-day-old domestic ducks and the time of *in ovo* 2,4-D herbicide spray application. The bars represent the mean relative bursa weights for the following exposure groups: early (day 6 of incubation) and late (day 21 of incubation). Error bars represent ± SD of the mean. *Mean bursa weights/body weights are significantly different between water control and low dose herbicide spray treatments ($P = 0.0434$).

3.4 Discussion

Analysis of chicken and duck eggs demonstrated measurable transfer of herbicide residues through the shell and into the embryo 24 hours after spraying. As expected, mean 2,4-D residue concentrations were higher in both chicken and duck eggs from the high dose (10X) groups than in eggs exposed to the recommended field rate of herbicide application (1X). Embryo residue concentrations in the chicken eggs (duck eggs not collected) increased from the day following exposure to 5 days after spraying, in both low and high dose groups. Mean concentrations in the 1X group increased from 0.6 to 2.2 ng/g, while 2,4-D residues in the 10X group increased from 27.4 to 374.5 ng/g during this time period. These findings are consistent with previous studies that have demonstrated the transfer of externally applied 2,4-D ester into bird embryos (Somers et al. 1974, Duffard et al. 1987, Castro de Cantarini et al. 1989, Várnagy 1999) and gradual uptake of the herbicide over the duration of embryonic development (Castro de Cantarini et al. 1989). The study performed by Castro de Cantarini et al. (1989) found that after fertile hens were topically exposed to 2,4-D ester *in ovo* on E0, the herbicide was detectable in the embryo by E5 and continued to increase in concentration throughout embryonic development. This observation indicates that risk of contaminant-induced adverse effects may continue to increase for at least several days after exposure.

This study employed a panel of immunotoxicity tests to evaluate the effects of *in ovo* exposure to 2,4-D on the developing avian immune system. Assays were chosen to assess cell-mediated immunity, humoral immune function (antibody production), and general immune system structure in exposed birds. The tests chosen to assess immune function and evaluate potential immunomodulation are standard assays validated by the National Toxicology Program in the USA, and organized into a tiered screening system for suspected immunotoxicants. Tests from Tiers I and II of the screening system are suitable for a general assessment of the immune system and a more comprehensive investigation of immunotoxic effect, respectively (Luster et al. 1992, Luster et al. 1993).

The immunomodulatory potential of exposure of developing chicken embryos to a commercial 2,4-D herbicide formulation was assessed by selected Tier I screening tests intended to provide information on two arms of the specific or adaptive immune system and an overview of the structural components of the immune system. After chicks reached 21 days of age, H/L ratios and relative lymphoid organ weights (spleen and bursa of Fabricius)

were measured, and primary and secondary immune organs (thymus, spleen, and bursa of Fabricius) were collected for histopathological evaluation. Changes in immune endpoints were assessed using herbicide treatment (high and low 2,4-D concentrations) and time of exposure (early and late incubation stages) as fixed factors of effect. Differences in these factors did not translate into significant effects in the final model analyses for H/L ratios, histopathological evaluation and relative spleen weights. Relative bursal weight of 21-day-old chicks was significantly associated with the different herbicide treatments ($P = 0.0006$). Mean bursal weight/body weight for birds exposed *in ovo* to the low concentration (recommended field application rate of 2,4-D) herbicide spray was significantly lower than the mean relative bursal weight of birds that received water spray (negative control, $P = 0.04$). The effect of the high concentration herbicide spray on bursal weight compared with controls approached significance ($P = 0.08$). In addition, mean relative bursal weights were significantly different between low dose and high dose herbicide spray treatments ($P = 0.0002$). Mean body weights for 21-day-old chicks among treatment groups were not significantly different, therefore lower relative bursal weights for chicks in the low dose herbicide group means that bursas in this group were actually smaller than the controls. In avian research it is common to evaluate relative organ to body weight ratios of primary and secondary lymphoid organs following exposure to an agent that may reduce their weights. Decreasing bursal to body weight ratios may predict a decreased antibody response and potential immunosuppression. While relative bursal weights were significantly lower in chicks in the low dose group, the opposite effect was observed in the high dose chicks, compared with the controls. This response may be a compensatory reaction to increasing herbicide exposure.

The Tier II tests used in the present study enabled evaluation of potential modulation of both the cell- and humoral-mediated immune systems of newly hatched chickens. Humoral-mediated immunity was assessed by measuring specific antibody production in response to BSA sensitization. Antibody production in response to antigenic challenge is a meaningful test of immune competence (Smits and Janz 2005). Primary and secondary anti-BSA antibody titres were measured in 14-day-old and 21-day-old chickens exposed to different concentrations of herbicide and at different periods of incubation. In this study, differences in 2,4-D dose and/or time of exposure did not affect antibody production in

newly hatched chickens. All exposure groups demonstrated typical primary and secondary humoral antibody responses to BSA immunization, and gave a positive reaction against BSA in the ELISA. Results similar to these have been found after environmental contaminant exposure in birds. In mallard ducks (*Anas platyrhynchos*), antibody titres against sheep red blood cells were unaffected by exposure to selenium (Fairbrother and Fowles 1990) or the polychlorinated biphenyl (PCB) mixture Aroclor 1254 (Fowles et al. 1997). Humoral immunity, may react variably to exposure to environmental stressors. Other immunotoxicity studies have demonstrated both enhancement and suppression of humoral immunity in birds exposed to metals, PCBs and pesticides. In some of the cases, antibody production differed between gender (Bunn et al. 2000, Smits and Bortolotti 2001, Singhal et al. 2003, Eeva et al. 2005). Although measurement of specific serum immunoglobulin levels is a reliable test of immune function, it is only one aspect of a very complex and redundant system. Normal antibody responses do not exclude the possibility of immune dysfunction. Therefore, additional assays to assess other components of the immune system are necessary for a thorough evaluation.

As a subtle measurement of the complex cellular reactions in cell-mediated immunity, the DTH test can be used to study the functionality of this response in animals exposed to immunotoxicants (Abbas 2005). The present study used the DTH test to assess potential changes in the cell-mediated immune system of chickens exposed *in ovo* to 2,4-D formulation spray. Using herbicide treatments (high and low concentrations of 2,4-D spray) and times of spray exposure (during either early or late stages of incubation) as contributing factors of effect, the DTH response in chickens was found to be statistically similar ($P > 0.05$) among all exposure groups. The DTH test has been employed in other avian immunotoxicity studies of environmental contaminants, with mixed results. The cell-mediated immune response of chickens was shown to be unaffected by a single exposure to lead at embryonic day 5. Negative outcomes were observed in birds exposed to lead during early stages of development, but inhibition of the DTH response was only demonstrated with lead exposure later in incubation (after embryonic day 12) (Bunn et al. 2000, Lee et al. 2001, Lee et al. 2002). These results show that the timing of toxicant exposure during embryonic development is significant, relative to immune system sensitivity as measured by the DTH test. Other studies of the immunotoxic effects of metals have also demonstrated the value of

the DTH test as a method to assess cell-mediated modulation of the avian immune system (Chen et al. 1999, McCabe et al. 1999), and a suitable biomarker for the assessment of xenobiotic-induced immunotoxicity (Bunn et al. 2000). For example, the DTH response to tuberculin was significantly depressed in selenium exposed mallards as compared to controls (Fairbrother and Fowles 1990).

To examine the potential effects of *in ovo* 2,4-D exposure in a representative waterfowl species, parallel experiments using the same battery of immunotoxicity assays were conducted with a domesticated strain of mallard duck (*Anas platyryhnchos*). As with the chicken model, changes in immune endpoints were assessed using the fixed factors herbicide treatment (high and low concentrations) and times of exposure (early and late incubation stages) to determine effects in newly hatched ducklings. Differences in these factors did not affect antibody response (as measured with the ELISA), the DTH test, histopathological evaluation and relative spleen weights. However, relative bursa weight was associated with time of spray exposure ($P = 0.04$). In addition, mean H/L ratios in blood from 21-day-old ducklings were significantly different between the groups treated with the high concentration of 2,4-D and controls ($P = 0.04$).

A variety of immune function assays were used in these experiments to evaluate the immunotoxic potential of an *in ovo* 2,4-D spray exposure in newly hatched chickens and ducks. There was little evidence that exposure to commercial 2,4-D formulation (at both the recommended field application concentration (1X) and a concentration representing a worst-case scenario exposure (10X)) at early and late incubation stages effected the immune components evaluated. Exceptions to this general observation include significant associations between herbicide treatment and relative bursal weight in chickens ($P = 0.0006$), and H/L ratio ($P = 0.11$) in ducks, as well as a significant association between time of spray exposure and relative bursal weight in ducks ($P = 0.04$).

In ducks, the H/L ratio was dose-dependent, and the difference became significant when the high dose 2,4-D treatment group was compared to the control group. An increase in the H/L ratio has been used in several bird species as an indicator of potential immunological stress from a variety of causes, including environmental contaminants (Grasman and Scanlon 1995, Bishop et al. 1998, Blanco et al. 2004).

Lymphoid organ/body weight ratios are routinely evaluated in immunotoxicity studies as a general measure of immune health. In avian species, changes in the weight of the bursa of Fabricius may forecast changes in B lymphocyte production and subsequent modifications of the humoral immune response (Pope 1991). Decreases in the relative bursa/body weight ratio have been demonstrated after contaminant (including pesticide) exposure in a number of studies (Bishop et al. 1998, Bosveld et al. 2000, Feyk et al. 2000, Garg et al. 2004). In the present study, lower relative bursa weights were observed in chickens exposed *in ovo* to the low concentration of 2,4-D spray. Since birds exposed to the high concentration of 2,4-D did not exhibit a similar change in mean bursa weight, it is unlikely that the decrease in weight was biologically relevant. Antibody production was not affected by 2,4-D exposure. In ducks, bursal/body weights were associated with time of spray exposures. In this instance, the groups of ducks that received spray treatment at a later incubation stage (day 21) had lower bursal/body weights than those exposed *in ovo* on day 6. There was no association between 2,4-D treatment and bursal weights. Therefore, the significant difference between times of spray exposures is not attributable to 2,4-D exposure at these times, and the reason for this observed difference or it's biological significance, are unknown.

Studies using *in ovo* exposure (including those using the chicken model) to evaluate the toxicity of other contaminants have demonstrated that embryo sensitivity to many toxicants is a function of embryonic development, and is consequently related to exposure time during incubation (Lee et al. 2001, Lee et al. 2002, DeWitt et al. 2005). The lack of effect on the DTH and antibody response observed in the present study indicates that *in ovo* 2,4-D exposure does not significantly impair cell mediated or humoral immunity in young birds, in spite of challenging the embryos at two times during development, with herbicide concentrations up to ten times recommended levels. Further *in ovo* studies herbicide exposure during other (e.g., earlier) developmental stages may be warranted. Although a number of other studies have evaluated the effects of contaminant exposure using the *in ovo* chicken model, few have compared times of embryonic exposures to the incidence of immunotoxic effects and consequences on avian health and development. This study has addressed that research gap in part, by using a novel exposure method to investigate the effects of a commonly used herbicide on the immune system of developing birds, and to

evaluate potential temporal differences of *in ovo* contaminant exposure at two periods during incubation.

CHAPTER 4

GENOTOXIC EFFECTS OF *IN OVO* BUCTRIL-M® EXPOSURE IN DOMESTIC CHICKENS (*GALLUS GALLUS*)

Abstract

Low tillage and fall seeding techniques are now routinely practiced on the Canadian prairies to conserve soil moisture and maintain topsoil. These practices often improved vegetative cover in the early spring for nesting waterfowl and upland game birds. The nesting period for these species often coincides with herbicide treatment for weed control in many important cereal crops. Therefore, eggs of ground nesting birds have the potential to be exposed during routine spray application. The herbicide formulation Buctril-M®, containing a 50:50 mixture of bromoxynil (4-cyano-2,6-dibromophenol) and MCPA (4-chloro-2-methylphenoxyacetic acid), is commonly used on the Canadian prairies for weed control. Previous studies indicate the potential for sublethal effects, including DNA damage, if developing birds are exposed to these herbicide components. The present study assessed the effects of *in ovo* exposure to Buctril-M® on the genetic integrity of newly hatched domestic chickens (*Gallus gallus*).

Fertile eggs were sprayed with the herbicide at either normal field application rates or at 10 times recommended rates on days 6 or 15 of incubation, to evaluate risks from herbicide exposure during early or late developmental stages, respectively. Control groups consisted of eggs sprayed with water only. Damage to genetic material was evaluated using two genotoxicity assays. The comet assay was used to measure DNA strand breaks in peripheral blood lymphocytes collected from 7-day-old birds, and flow cytometry was used to evaluate DNA content variability in circulating erythrocytes collected from 21-day-old birds. In the comet assay, DNA strand breaks are detected as fragments or uncoiled loops that migrate away from nuclear DNA during electrophoresis to form a measurable "tail", and damage is quantified using three measurements: comet tail length, percent DNA in

the tail, and tail moment (tail length multiplied by the % DNA in the tail). Flow cytometry can be used to estimate the variability in DNA content among a specific population of cells. Variability in DNA content among cells increases as a result of unequal distribution of genetic material during mitosis, as may occur following exposure to clastogenic agents. DNA content differences (measured in 10,000 erythrocyte nuclei) within individuals are usually reported as the half peak coefficient of variation (HPCV).

A mixed linear statistical model was used to analyze the relationship between herbicide exposure and genotoxicity. Herbicide treatment with Buctril-M® and time of exposure during incubation were accounted for as fixed factors. The results of this study demonstrated that *in ovo* spray exposure to Buctril-M® does not have a significant effect on genetic integrity in domestic chickens, as measured by the comet assay and flow cytometry ($P > 0.05$). Early or late herbicide exposure was also not associated with DNA damage as assessed by the comet assay. The significant association ($P = 0.0210$) observed between time of spray exposure and the variability in DNA content of erythrocytes in 21-day-old chicks (increased variability in cells from birds sprayed on day 6 of incubation) was not attributable to Buctril-M® treatment at these timepoints.

4.1 Introduction

In recent years, significant efforts have been made to reduce the negative environmental impacts of agriculture in Canada by implementing techniques that follow the concepts of conservation agriculture. Conservation agriculture strives to achieve sustainable (while still profitable) crop production through the application of practices to minimize soil disturbance and therefore topsoil and moisture loss by increasing year-round crop cover. Conservation strategies that can be adopted easily by individually managed farming systems include reducing soil tillage of cropland and planting winter cereals. Fields that receive low to no till and/or are planted with winter cereals (providing plant cover in the spring) help to preserve soil and water resources and maintain wildlife habitat and biodiversity on the prairies. Many species of birds are attracted to the nesting habitat provided by the plant stubble in minimally tilled fields and early emerging winter cereal crops (Lokemoen and Beiser 1997). However, a disadvantage of this habitat-friendly conservation strategy is the increasing reliance of farmers on agrochemicals for primary weed control.

Conventional tillage methods effectively control the majority of persistent weed growth in cropland. However, with the adoption of conservation tillage practices (less mechanical weed removal), cultivation operations are often replaced with intensified herbicide application to control weeds. Therefore, herbicide application rates are typically higher in low or no till fields (Gebhardt et al. 1985, Campbell 1999, FAO 2006). Because typical spraying periods for spring weed control overlap with the nesting period of many species of ground-nesting waterfowl and upland game birds, the risk of egg exposure is significant. The commercial mixture, Buctril-M® is one of the top herbicides applied to winter cereal crops and fields receiving minimal till (Fowler 2002). Buctril-M® is a 50:50 mixture of bromoxynil (4-cyano-2,6-dibromophenol) and MCPA (4-chloro-2-methylphenoxyacetic acid). The potential long-term effects of low rates of exposure of wildlife, including avian species, to the herbicides in this product have yet to be investigated, especially when exposure occurs during embryonic development. Therefore, because conservation practices may increase the risk of embryonic exposure to Buctril-M® in critical prairie pothole breeding areas, it is important to investigate potential sublethal effects of this herbicide formulation on developing birds.

Interest in genotoxic effects of exposure to environmental contamination is rapidly growing as part of a desire to better understand subtle mechanisms of toxicity in wildlife. The effects of genotoxic substances (e.g., chemical genotoxicants, ultraviolet light, and ionizing radiation) and their potential biological damage to DNA has been studied for many years by human health researchers. Genetic changes observed in exposed human populations have provoked concern about the potential for similar effects on the integrity of genetic material of wildlife species in the natural environment. There is a need to investigate and document subtle genotoxic effects and changes to the genetic integrity of organisms exposed to environmental contaminants (Anderson et al. 1994). Genotoxic agents may produce adverse effects at the cellular level, resulting in structural changes to DNA (e.g., strand breaks, adducts, base modifications, etc.). These changes to DNA can be used as biomarkers of genotoxicant exposure, in place of epidemiological monitoring of genotoxicological diseases, such as carcinogenesis (Shugart 1999, Shugart et al. 2003). The purpose of a useful biomarker is to be able to reveal whether organisms have been exposed to potentially toxic substances, and to indicate the magnitude of the organism's response to exposure, preferably

before more severe effects appear. Furthermore, studying markers of genotoxic effects may ultimately reveal other population-level effects that result from critical contaminant-induced genetic changes (Kleinjans and van Schooten 2002, Shugart et al. 2003).

Toxicological data concerning the potential effects of Buctril-M® exposure on wild birds and mammals, is limited. Much of the research that has been performed has involved determining the risk of Buctril-M® to aquatic invertebrates and fish (Buhl et al. 1993a, Buhl et al. 1993b, Morgan and Brunson 2002) or evaluating effects of the herbicide formulation using standard toxicity tests with laboratory animal models (e.g., LD_{50} = 368 mg/kg in the rat) (Bayer CropScience Inc.). Although studies evaluating the toxic effects of the Buctril-M® mixture on terrestrial wildlife species are few, some work on mammalian and avian development has been done to determine the relative toxicities of the individual herbicide components of Buctril-M®, bromoxynil (heptyl and octyl esters) and MCPA (2-ethyl hexyl ester).

Bromoxynil is a nitrile herbicide that is used for post-emergent control of broadleaf weeds through inhibition of photosynthesis. It is frequently tank mixed or commercially formulated with other herbicides such as 2,4-D (2,4-dichlorophenoxyacetic acid) and MCPA (Saskatchewan Agriculture and Food 2006). In acute toxicity tests, bromoxynil is highly to moderately toxic to many avian species, including pheasants, hens, quail, and mallard ducks, with a dietary LC_{50} as low as 50 mg/kg (Kidd and James 1991). In chronic toxicity tests, it has been shown to affect bone development in rats and mice (evidence of supernumary ribs in both species), and is a suspected teratogen (Rogers et al. 1991, Chernoff et al. 1991). The sublethal toxic effects of bromoxynil are not as well known as other popular herbicides, and although bromoxynil has not been shown to be genotoxic or carcinogenic in acute and chronic toxicity tests, potential subtle effects on developing avian embryos appear reasonable.

The toxicity of chlorophenoxy herbicide compounds has been extensively studied. Since the late 1940s, this class of herbicides (acting as auxin hormone mimics in plants) has seen continuous use in agriculture for the control of broadleaf weeds and woody plants. Along with 2,4-D and 2,4,5-trichlorophenoxy-acetic acid (2,4,5-T), MCPA is one of the most common chlorophenoxyacetic acid herbicides. It is routinely used on the Canadian prairies as the main active herbicide ingredient in formulated sprays, or as a popular component in

herbicide mixtures with 2,4-D, dicamba, mecoprop, and bromoxynil (Saskatchewan Agriculture and Food 2006).

The potential genotoxicity of MCPA has been evaluated in a number of *in vitro* and *in vivo* assays using bacterial, mammalian and avian test systems. A comprehensive review of the literature evaluating the genotoxicity of MCPA was performed by Elliot (2005). The herbicide is mildly to non-mutagenic in standard *in vitro* bacterial and mammalian mutation assays. MCPA was found to cause limited cell cycle delay, in conventional cell metaphase analysis of peripheral human lymphocytes treated *in vitro*, although higher doses (2000 μg/ml) caused increases in aberrant cells, accompanied by increased cytotoxicity. In cytogenic studies involving measurement of sister chromatid exchange (SCE), MCPA was reported to produce small, but statistically significant, increases in SCE in CHO cells treated *in vitro* (Linnainmaa 1984). The herbicide was also examined for cytogenetic effect endpoints, including micronucleus formation, chromosomal aberrations and SCEs, in a range of *in vivo* assays. Results indicated no tendency to cause micronucleation, therefore MCPA was deemed non-clastogenic in those systems. In mammalian models, MCPA appears to act as a peroxisome proliferator, increasing either the size or amount of peroxisomes in the hepatocytes of Chinese hamsters (Vainio et al. 1982), and was shown to increase sister chromatid exchange in mammalian cells (Linnainmaa 1984). Similarly, in avian models, MCPA also acts as a peroxisome proliferator, inducing sister chromatid exchanges in embryonic chromosomes (Arias 1992). In summary, despite the relatively mild acute toxicity of MCPA in wildlife species, and the evidence that it is not overtly genotoxic *in vivo* (Elliot 2005), it is apparent that this herbicide has demonstrated the ability to cause subtle changes to the genetic material of mammalian and avian cells. In the present study, the potential genotoxic effects of MCPA and bromoxynil (Buctril-M®) on developing bird embryos was evaluated using the comet assay and flow cytometric analysis to measure potential DNA strand breaks and clastogenic damage, respectively, in newly hatched domestic chickens.

DNA strand breakage occurs at baseline levels under natural, physiological conditions in all cells. However, exposure to genotoxic agents may cause a significant increase in the frequency and/or severity (i.e. increased unrepairable lesions) of DNA damage (Shugart and Theodorakis 1998). The alkaline comet (single cell gel

electrophoresis) assay is used to detect various types of strand breaks (single, double, and alkali-labile sites expressed as strand breaks) in DNA, which may be indicative of contaminant exposure (Brendler-Schwaab et al. 2005). In this assay, breaks become visible after cellular suspensions undergo cell lysis and DNA unwinding, followed by electrophoresis which causes uncoiled DNA or DNA fragments to migrate out of the nucleus, forming a measurable "comet tail". After comets are visualized with fluorescent dye, the extent of DNA damage can be quantified by measuring the size and fluorescent intensity of the tail (Tice et al. 2000). DNA strand breakage in the comet assay is usually based on measuring the amount of DNA in the tail (the damaged DNA) as a proportion of the total nuclear DNA. There is no consensus as to the best metric, but the most commonly used include comet tail length (measured from the leading edge of the head to the tip of the tail, in μm), tail DNA content (% DNA in the tail), and tail moment (tail length multiplied by the % DNA in the tail).

Increases in DNA fragmentation as a result of contaminant exposure has been documented in a number of studies monitoring genotoxic effects of xenobiotics in various species, including mammals, amphibians, and birds (Pandrangi et al. 1995, Nacci et al. 1996, Ralph et al. 1996, Clements et al. 1997, Pastor et al. 2001a & 2001b, Ateeq 2005). Studies using the comet assay have demonstrated the association of increasing genetic damage to increases in the size and stain intensity of the comet tail. Therefore, assessment of structural damage to DNA based on measurement of strand breakage has been shown to be a valid biomarker of genotoxicity.

Structural alterations to DNA that remain unrepaired may produce irreversible chromosomal changes within a cell, and these changes may be heritable. During the process of DNA replication and cell division, clastogenic damage may alter the proper (i.e., equal) allocation of chromosomes into daughter cells, resulting in abnormal cells that contain different amounts of DNA. The DNA content of a population of cells can be measured using flow cytometry. The degree of DNA content variability among the population of cells (as measured by either the coefficient of variation, CV, or half-peak coefficient of variation, HPCV) gives an indication of the extent of clastogenic damage. A number of wildlife field studies have used flow cytometry to investigate the impacts of environmental genotoxicants. These studies have demonstrated that increased variability of DNA content is a useful

biomarker for detecting subtle changes in the genetic integrity of wild species (Bickham et al. 1988, George et al. 1991, Bickham et al. 1992, Bickham et al. 1994, Custer et al. 1994, Lamb et al. 1995, Lowcock et al. 1997, Custer et al. 2000, Matson 2004). Flow cytometry has demonstrated chromosomal damage in birds exposed to petroleum hydrocarbons (Custer et al. 1994), radioactive waste (George et al. 1991), and polycyclic aromatic hydrocarbons (Custer et al. 2000, Matson et al. 2004). However, to the author's knowledge, there are no published reports of the use of this technique to assess chromosomal damage in birds exposed to pesticides.

The purpose of this study was to investigate whether *in ovo* exposure to the commercial herbicide formulation Buctril-M® was associated with changes to the genetic integrity of domestic chickens (*Gallus gallus*), which was chosen as a surrogate for wild upland game birds. Potential genotoxic effects of herbicide exposure were assessed using the comet assay to evaluate increased DNA strand breaks in isolated peripheral blood lymphocytes from 7-day-old hatchlings, and flow cytometry to measure chromosomal damage in circulating erythrocytes from 21-day-old hatchlings. The *in ovo* herbicide exposure design was intended to simulate a scenario in which eggs of upland game birds are sprayed with herbicide during weed control operations at different times of incubation. The results of this study will help to determine the subtle impacts of Buctril-M® on different stages of avian embryonic development.

4.2 Materials and Methods

4.2.1 Animal Model

Fertile chicken (*Gallus gallus*) eggs from a White Leghorn/Brown Leghorn cross were incubated at 37.5°C and approximately 80% humidity in circulated air incubators (Hova-Bator®, G.Q.F. Manufacturing Co., Savannah, Georgia, USA) until 1 day post-hatch (about day 23). Automatic egg turners (Hova-Bator®, G.Q.F. Manufacturing Co., Savannah, Georgia, USA) were used for the first 18 days of incubation. On day 19 of incubation, egg turners were removed and eggs were placed on the wire floor of the incubator. Humidity was increased to approximately 90% in accordance with hatching requirements. Chicks were transferred to heated brooders with raised wire floors at one to two day(s) of age, and maintained on *ad libitum* chick starter and fresh water for the duration of the study.

4.2.2 Herbicide Spray Exposures

Fertile chicken eggs (4 replicate groups each of 120 eggs) were randomly assigned to one of six incubators (20 per incubator), and each incubator was randomly assigned to a specific treatment. In avian embryos, different types of developmental effects may be attributed to genotoxicant insult during critical periods of embryonic development. In order to account for time-specific vulnerability of the embryos, eggs were exposed to the herbicide during either an early (day 6) or late (day 15) stage of incubation (DeWitt et al. 2005).

Eggs were sprayed with the commercial Buctril-M® (Bayer CropScience Inc., Calgary, Alberta, Canada) formulation at one of two different concentrations: 1) Low dose groups (early and late) were sprayed with Buctril-M® at the recommended field application rate for winter wheat (0.55 L ai ha^{-1}) (SAFRR 2005). This was equivalent to 4.94 ml of formulated Buctril-M® per litre of aqueous spray solution. 2) High dose groups (early and late) were sprayed with 49.4 ml of formulated Buctril-M® per litre, equivalent to 10 times the recommended concentration of herbicide, to simulate a worst-case exposure. Additional groups of eggs (early and late) were sprayed at the same time points with water only to act as negative control groups. The spray treatments were applied using an agricultural field spray simulator (Research Instrument Company, Guelph, Ontario, Canada) (Figure 2-1) at the Agriculture and Agri-Foods Canada Research Centre in Saskatoon, Saskatchewan, to reproduce actual field application conditions as closely as possible. Eggs were masked prior to spraying to ensure that every egg received similar amounts of herbicide (Figure 2-1). The six treatment groups comprising each replicate were: high Buctril-M® dose (early and late incubation exposures); low Buctril-M® dose (early and late); and negative control (water only, early and late). The four replicate experiments were spaced about two weeks apart.

4.2.2.1 Quantifying Herbicide Exposure

Herbicide deposition on the surface of the eggs was quantified in a separate study in which surplus eggs of both species were sprayed with fluorescein dye solution (10% (w/w) fluorescein sodium salt in water) at the same application rate as the herbicide treatments. The amount of fluorescein dye deposited on the exposed portion of the eggs was determined by rinsing the eggs to remove the dye, and determining the amount of fluorescein in the rinsate. The rinsate fluorescence was measured at 498 nm using a spectrofluorometer (Shimadzu RF-1501, Shimadzu Corporation, Columbia, MD, USA).

4.2.3 Sample Collection

Whole blood is not generally used with the comet assay in birds because > 80% of the cells exhibit the "ghost cell" appearance associated with apoptosis, which is presumably due to degraded and functionally inert DNA/RNA within nucleated, mature erythrocytes (Knopper and McNamee 2006). Therefore, the comet assay was performed using isolated peripheral blood lymphocytes from 7-day old chicks. Immediately prior to blood collection, a subset of five birds per treatment group was randomly selected for the assay, and body weight was measured to the nearest gram using an electronic balance (Mettler PK 4800). Blood samples were collected from the jugular vein with a heparinized syringe into heparinized Microtainer® tubes and kept on ice, protected from light, until analysis, which occurred within 2 hours of collection. At least 250 µl of whole blood was required from each bird to obtain sufficient numbers of lymphocytes for the comet assay. Flow cytometry was performed on peripheral erythrocytes from all 21-day old chicks. Blood was collected from the jugular vein with a heparinized syringe into heparinized Vacutainer® tubes, and a 500 µl aliquot of each sample was mixed in 1.0 ml cryovials (Nalge Nunc International, Rochester, NY, USA) with an equivalent volume of chilled citrate buffer, consisting of 250 mM sucrose, 40 mM trisodium citrate, and 5% v/v DMSO, adjusted to pH 7.6 with 1.0 M citric acid (BDH, Toronto, ON, Canada). Samples were immediately frozen in liquid N_2, and stored at -80°C until flow cytometric DNA analysis could be performed.

After collection of blood for flow cytometry on day 21, birds were euthanized by cervical dislocation. The use of animals in this study was approved by the University of Saskatchewan Committee on Animal Care and Supply. Birds were housed, handled, and sacrificed in accordance with guidelines established by the Canadian Council on Animal Care.

Unless otherwise noted, all reagents and chemicals were purchased from either EMD Chemicals Inc. (Gibbstown, NJ, USA) or Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada), while laboratory disposables were obtained from VWR International (Mississauga, ON, Canada).

4.2.4 Comet Assay

The comet assay was performed using isolated peripheral lymphocytes, according to procedures outlined by Knopper (2005). This standard method is based on techniques

optimized by McNamee et al. (2000) and originally developed by Singh et al. (1988). The agarose solution consisted of 0.75% w/v DNA grade, low melting point (LMP) agarose (Fisher Biotech, Fairlawn, NJ, USA) in phosphate buffered saline (PBS) composed of 58 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 68 mM NaCl, pH 7.4. The lysis buffer (pH 10.0) was prepared with 2.5 M NaCl, 100 mM tetrasodium EDTA, 10 mM Tris base, and 1% w/v *N*-lauryl sarcosine, with the addition of 1% v/v Triton X-100 to required volume 30 min prior to use. The alkaline unwinding (electrophoresis) buffer was prepared fresh on the day of the experiment, with 0.3 M NaOH, 10 mM tetrasodium EDTA, 0.1% w/v 8-hydroxyquinoline, and 2% v/v DMSO, adjusted to pH 13.1 with concentrated NaOH or HCl.

Lymphocytes were isolated from whole blood samples using Ficoll-Paque Plus® (Amersham Biosciences Corporation, Piscataway, NJ, USA), following a modification of the procedure recommended by the manufacturer. Three 15 ml Falcon® conical centrifuge tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were numbered and labeled for each blood sample, with 250 µl PBS added to tube #1, 3.0 ml Ficoll-Paque Plus® added to tube #2, and 1.0 ml PBS added to tube #3. Tubes were stored overnight at 4°C. All subsequent steps were performed under reduced light conditions within one hour after blood collection. Whole blood (250 µl) was added to tube #1 to make a 1:1 suspension of blood in PBS. Using a Pasteur pipette, the contents of tube #1 were mixed and carefully layered on top of the Ficoll-Paque Plus® gradient in tube #2. To keep the "layers" separate, the tube and pipette were held at a 45° angle, and the pipette tip was kept about 5 mm above the Ficoll-Paque Plus® as the blood was expelled. All #2 tubes were centrifuged for 30 min at 2000 rpm (Beckman J-6B, Beckman-Coulter, Mississauga, ON, Canada). After centrifugation, the white blood cell (WBC) layer (buffy coat) was withdrawn with a pipette, and was added to tube #3. After mixing, the tubes were centrifuged for 10 min at 2000 rpm, and the supernatant was poured off. The WBC pellet was resuspended in a known volume (usually 500 µl) of PBS, and placed on ice. Cell viability was calculated to maintain consistency among samples (as compared to the control), and to assess cytotoxicity in the cell suspension to determine the cause (genotoxic or otherwise) of cell damage (Tice et al. 2000, Knopper 2005). Lymphocyte viability was assessed using Trypan blue exclusion within one hour of the assay. A 50 µl aliquot of the WBC suspension was added to 50 µl of Trypan blue working solution, consisting of a 1/40 dilution of Trypan blue stock in a 1% v/v acetic acid

solution in saline. Viable cells were counted with a hemocytometer and light microscope at 40X. Only samples with > 90% lymphocyte viability were used in the comet assay.

All steps of the comet assay were performed in subdued light. A 30 µl aliquot of the purified WBC suspension was added to 270 µl of liquefied 0.75% agarose and gently mixed. Aliquots (120 µl each) of the cell/agarose mixture were then cast in duplicate into individual wells of a two-well Lab-Tek® chamber (Nalge Nunc International, Rochester, NY, USA) affixed to GelBond® film (FMC Bioproducts, Rockport, ME, USA). Each piece of film supported three chambers (three different samples, in duplicate). Internal control samples were also cast simultaneously into four wells, to represent negative and positive controls, designed to assess assay performance and comet formation, respectively. Once the agarose solidified (approximately two min), the Lab-Tek® chambers were carefully removed, leaving the agarose-embedded cells attached to the films. Positive control films were exposed for five min to ice-cold, freshly prepared 1 mM H_2O_2 in PBS. Remaining films were each immersed in 50 ml ice-cold lysis buffer, and maintained at 4°C in the dark for 60 min. After lysis, films were gently rinsed with distilled water (ddH_2O) and placed into 50 ml fresh electrophoresis buffer for 30 min at room temperature to allow the DNA to unwind. Electrophoresis was subsequently performed in chilled Hoefer HE33 gel electrophoresis units (Hoefer Scientific Instruments, San Francisco, CA, USA) containing 220 ml electrophoresis buffer. Electrophoresis gel units were powered by a Thermo EC570-90 power unit (Thermo Electron Corporation, Waltham, MA, USA), and films were run for 20 min at 19 V (~1.5 V/cm constant voltage, >300 mA). After films were electrophoresed, they were placed in 50 ml 1 M ammonium acetate neutralization solution for 30 min, then transferred to 95% ethanol for two hours to dehydrate before air-drying overnight. Dry films were labeled and stored in envelopes until imaging analysis.

Image analysis was performed on one set of samples and all control gels. GelBond® films were cut into three strips, each containing one sample. Individual films were stained for 10-15 min in a 1/10,000 dilution of stock SYBR Gold® (Molecular Probes, Eugene, OR, USA) in ddH_2O. Films were double rinsed in water, placed onto a glass microscope slide (gel side up), covered with a cover slip (22 x 50 mm), and gently pressed with a cloth to remove excess water and form a seal. Stained slides were examined with a Zeiss Axioplan 2 fluorescent microscope (Carl Zeiss Microscopy, Jena, Germany), and comet images were

captured with a QImaging Retiga™ 1300 digital CCD monochrome camera (QImaging, Vancouver, BC, Canada). A minimum of 50 cells per slide were scored for DNA migration, and comets were analyzed using Komet version 5.5 comet assay software (Kinetic Imaging, Nottingham, UK) at 430x magnification. The degree of damage was quantified using three different metrics: comet tail length (measured from the leading edge of the head to the tip of the tail, in μm), tail DNA content (% DNA in the tail), and tail moment (tail length multiplied by the % DNA in the tail). Outlier values greater than four standard deviations from the sample mean of the 50 cells were identified and removed. All sample gels were scored without knowledge of the treatment group.

4.2.5 Flow Cytometric DNA Analysis

Flow cytometric DNA analysis was performed on erythrocytes from whole blood samples collected from 21-day-old chicks to determine cell to cell variability in DNA content. The methods used for DNA content analysis followed those previously described by Vindeløv and Christiansen (1994). Unless noted, all solutions (pH 7.6) were prepared up to a week prior to the start of the experiment, and stored at -20°C until needed. A stock solution containing 3.4 mM trisodium citrate, 0.1% v/v IGEPAL CA-630, 1.5 mM spermine tetrachloride, and 0.5 mM Tris base, was used to prepare the remaining solutions, and was kept at 4°C. Solution A consisted of trypsin (30 mg/L stock) and Solution B contained trypsin inhibitor (500 mg/L stock) and ribonuclease A (100 mg/L). The stain solution contained 3.3 mM spermine tetrahydrochloride and propidium iodide (416 mg/L stock), and was stored in the dark at -20°C.

Samples were sorted prior to processing in order to ensure that each batch of samples analysed on a given day contained a representative from each treatment. Samples from each treatment were randomly chosen to avoid experimental bias. Frozen samples of whole blood were thawed rapidly at room temperature, and prepared for flow cytometric analysis as follows. A clean nuclear suspension was obtained by homogenizing 2 μl of the blood mixture with 50 μl of citrate buffer and 450 μl of Solution A in a microcentrifuge tube, and allowing the samples to sit for 10 min at room temperature. A 375 μl aliquot of Solution B was added to each sample in the microcentrifuge tubes, followed by another 10 min incubation at room temperature. The RNase A component of Solution B degrades double-stranded RNA, leaving only DNA to take up the fluorescent dye during the final step. After

10 min, the nuclear suspension was pipetted through a 37 μm mesh nylon filter cloth (Cole Parmer, Vernon Hills, IL, USA) into a 12x75 mm Falcon™ tube to remove as much cellular debris as possible. Finally, 375 μl of propidium iodide (PI) solution was added to each Falcon™ tube, and incubated for at least 15 min on ice. Samples were analysed on the flow cytometer within two hours of staining.

Nuclear fluorescence was measured on a Coulter Epics Elite® ESP flow cytometer (Beckman Coulter Canada Inc., Mississauga, ON, Canada). Instrument alignment and focus were set with fluorospheres (Flow-Check™, Beckman Coulter) each day, prior to sample analysis. Cells were analyzed at a rate averaging 200-300 cells per second to ensure a thin stream of cells intersecting the laser in a single path. The PI stain was excited using the 488 nm line of an argon ion laser. Fluorescence emission values were measured and plotted as histograms using Expo32® acquisition and analysis software (v.1.2, Beckman Coulter) to estimate the mean and standard deviation of the DNA content in each sample. Ten thousand nuclei in the G1 phase were measured (PMT3 linear vs. PMT3 peak as parameters) from each sample, and, using the histograms generated, the full peak CV (standard deviation/mean x 100, expressed as a percent) and half peak CV (HPCV) were calculated. CV and HPCV describe the width of the histogram peak (DNA content), and therefore represent the variability in cell DNA content. A wider peak results in an increased CV or HPCV, indicative of greater chromosomal damage.

4.2.6 Statistical Analysis

Descriptive statistics for all outcomes were compiled using SPSS (v.14.0, SPSS Inc., Chicago, IL, USA) and SYSTAT (v.11.0, SPSS Inc., Chicago, IL, USA). Summaries are reported for the subset of animals used for the comet assay (N=88) and for the entire set of animals assessed for flow cytometry (N=199). The normal distribution of all assay variables was assessed using the Shapiro-Wilk test for normality. All comet parameters were $\log_{10}$ transformed to attain normality if the p-value given by this test was low (i.e. < 0.5). Correlation (Pearson's r) among comet measurements ($\log_{10}$ mean comet tail DNA, tail moment, and tail length) was also determined.

The association between exposure and genotoxicity was analyzed using a mixed linear model (PROC MIXED in SAS v.8.2 for Windows, SAS Institute, Cary, NC, USA). Incubator and experimental group were included as random effects to account for clustering

of the observations as a result of separate incubator designations and replicated experiments, respectively. First, time of exposure (early or late incubation stage) and then herbicide treatment level (high, low concentration and water control) were assessed as fixed effect factors in a model including the random effects. Where time of exposure and herbicide treatment were both potentially important factors ($P < 0.25$), both were included in a model to assess confounding effects. Variables were retained in the final model if they were significant ($P < 0.05$), or acted as important confounders (i.e. adjustment for the variable changed the other coefficient by more than 10%). If both time of exposure and herbicide treatment were significant ($P < 0.05$), the model was then tested for interaction.

4.3 Results

Herbicide deposition on the surface of the eggs was quantified in a separate study in which fluorescein dye was sprayed onto masked eggs, eggs were rinsed, and the amount of fluorescein in the rinsate was measured using a spectrofluorometer. Mean doses of the Buctril-M® herbicide deposited onto masked eggs were calculated for low and high dose exposure groups, and reported in Table 4-1.

Descriptive statistics for the comet assay outcome variables are summarized in Table 4-2. For all comet metrics, simple comparisons of the assay outcomes from herbicide treated birds to those of the control group revealed only slight differences. Similarities among the variables were reinforced with correlation analysis using the Pearson's coefficient *r*. There was strong correlation among all comet measurements (Table 4-3), with all correlation values showing significance at the 0.01 level.

Table 4-4 summarizes the univariate comparisons between the fixed factors herbicide treatment and time of exposure, and all comet variables, in chicken lymphocytes. For comet tail moment (tail length multiplied by the % DNA in the tail) only time of exposure ($P = 0.14$) was considered a potentially important factor in the initial model ($P < 0.25$), therefore neither factor was included in a final model. For the tail DNA content (% DNA in the tail) and comet tail length, herbicide treatment and exposure time were not considered to be important factors in the initial model ($P > 0.25$). Interestingly, mean values for comet tail DNA content decreased with increasing herbicide concentration (as compared to the control group). However, this difference was not significantly different on further analysis using the final statistical model. Figures 4-1 and 4-2 show that the fixed effects factors associated with

Buctril-M® exposure (both herbicide concentrations and times of spray exposure) do not have significant effects on genetic integrity as measured by the comet variable tail DNA content.

Descriptive statistics for the flow cytometry variable half-peak coefficient of variation (HPCV) are listed in Table 4-5 for all exposure groups. HPCV values were $\log_{10}$ transformed to attain a normal distribution of the data. From the initial analysis with the univariate comparison model (Table 4-6), only time of exposure was associated with HPCV of erythrocyte DNA content ($P = 0.02$). Since herbicide treatment was not an important factor in the final model, the association between spray exposure and HPCV is not attributable to Buctril-M® exposure, and the biological significance of the difference between DNA variability at early and late timepoints during incubation is unknown. Figure 4-3 shows the lack of association between treatment as a fixed effect factor and HPCV. The significant difference between the HPCV values of birds exposed to spray treatment during early incubation compared with birds exposed late in incubation is demonstrated in Figure 4-4. Chicks that hatched from eggs sprayed on day 6 of incubation had higher mean $\log_{10}$ HPCV values than chicks exposed on day 15 of incubation.

Table 4-1. Buctril-M® application rates, spray solution concentrations, and actual doses of active ingredients deposited on chicken eggs in low (1X) and high (10X) exposure groups.

Herbicide Exposure	Application Rate[1] (L ai/ha)	Concentration[2] (ml/L)	Dose Deposited[3] (µg ai/egg)
1 X Buctril-M® (Low Dose)	0.55	4.94	80.8
10 X Buctril-M® (High Dose)	0.55	49.40	785.6

[1] Maximum safe application rate of herbicide on wheat crops (litres of active ingredient/hectare) (SAFRR 2005).
[2] Concentration of formulated Buctril-M® (ml) in water spray solution (L).
[3] Estimate based on fluorescein dye retention

Table 4-2. Descriptive summaries of comet assay outcomes (log_{10} transformed to attain normality) using lymphocytes isolated from the blood of 7-day-old domestic chickens previously exposed *in ovo* to Buctril-M® formulation spray. Summaries are provided for comet tail DNA (a), comet tail moment (b), and comet tail length (c).

(a) **Log_{10} Tail DNA**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	16	0.968	0.138	0.035	0.939	0.866	1.079
	Late[2]	16	0.956	0.122	0.031	0.990	0.892	1.041
1 X Buctril-M® (Low Dose)	Early	13	0.884	0.127	0.035	0.851	0.816	0.981
	Late	16	0.982	0.165	0.041	1.003	0.797	1.073
10 X Buctril-M® (High Dose)	Early	12	0.895	0.113	0.033	0.897	0.822	0.995
	Late	15	0.892	0.180	0.046	0.942	0.743	1.052

(b) **Log_{10} Tail Moment**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early	16	-0.055	0.191	0.048	-0.098	-0.198	0.126
	Late	16	-0.050	0.194	0.049	-0.016	-0.079	0.092
1 X Buctril-M® (Low Dose)	Early	13	-0.170	0.206	0.057	-0.213	-0.289	0.048
	Late	16	0.001	0.264	0.066	0.054	-0.230	0.176
10 X Buctril-M® (High Dose)	Early	12	-0.186	0.207	0.060	-0.170	-0.343	-0.057
	Late	15	-0.141	0.256	0.071	-0.131	-0.363	0.113

(c) **Log_{10} Tail Length**

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early	16	1.100	0.138	0.034	1.075	1.027	1.169
	Late	16	1.085	0.151	0.038	1.109	1.008	1.191
1 X 2,4-D (Low Dose)	Early	13	1.041	0.141	0.039	1.029	0.957	1.123
	Late	16	1.133	0.170	0.043	1.164	0.983	1.263
10 X 2,4-D (High Dose)	Early	12	0.994	0.134	0.039	1.005	0.857	1.090
	Late	15	1.038	0.217	0.056	1.005	0.945	1.269

[1] Day 6 and [2] Day 15 of incubation

Table 4-3. Correlation (Pearson's coefficient, *r*) among comet assay outcomes (log_{10} transformed to attain normality) for control (water), low dose (1X Buctril-M®), and high dose (10X Buctril-M®) treatment groups, at both early (incubation day 6) and late (incubation day 15) exposure times. Correlation tables are given for the following treatment groups: Control Early (a), Control Late (b), Low Dose Early (c), Low Dose Late (d), High Dose Early (e), and High Dose Late (f).

(a) **Control Early**; N = 16	**Comet Tail DNA**	**Comet Tail Moment**	**Comet Tail Length**
Comet Tail DNA	1.000	0.921*	0.795*
Comet Tail Moment	0.921*	1.000	0.912*
Comet Tail Length	0.795*	0.912*	1.000
(b) **Control Late**; N = 16			
Comet Tail DNA	1.000	0.932*	0.921*
Comet Tail Moment	0.932*	1.000	0.951*
Comet Tail Length	0.921*	0.951*	1.000
(c) **Low Dose Early**; N = 13			
Comet Tail DNA	1.000	0.974*	0.884*
Comet Tail Moment	0.974*	1.000	0.924*
Comet Tail Length	0.884*	0.924*	1.000
(d) **Low Dose Late**; N = 16			
Comet Tail DNA	1.000	0.954*	0.911*
Comet Tail Moment	0.954*	1.000	0.959*
Comet Tail Length	0.911*	0.959*	1.000
(e) **High Dose Early**; N = 12			
Comet Tail DNA	1.000	0.941*	0.896*
Comet Tail Moment	0.941*	1.000	0.944*
Comet Tail Length	0.896*	0.944*	1.000
(f) **High Dose Late**; N = 15			
Comet Tail DNA	1.000	0.969*	0.916*
Comet Tail Moment	0.969*	1.000	0.945*
Comet Tail Length	0.916*	0.945*	1.000

* Correlation value is significant at the 0.01 level (2-tailed)

Table 4-4. Summary of univariate comparisons between the fixed effect factors herbicide treatment and time of exposure, and comet tail DNA (a), comet tail moment (b), and comet tail length (c) in isolated blood lymphocytes from 7-day-old domestic chickens previously exposed *in ovo* to Buctril-M® formulation spray.

(a) **Comet Tail DNA**	**Regression coefficient (β)**	**95% Confidence Intervals for β**		***P* value**
		Lower	**Upper**	
Herbicide Treatment				
- High Dose[1]	-0.056	-0.130	0.019	0.14
- Low Dose[2]	-0.016	-0.089	0.057	0.66
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.027	-0.088	0.034	0.38
- Late[5]	Reference	-	-	-
(b) **Comet Tail Moment**				
Herbicide Treatment				
- High Dose[1]	-0.081	-0.197	0.035	0.17
- Low[2]	-0.012	-0.125	0.101	0.83
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.069	-0.163	0.024	0.14
- Late[5]	Reference	-	-	-
(c) **Comet Tail Length**				
Herbicide Treatment				
- High Dose[1]	-0.051	-0.131	0.028	0.20
- Low Dose[2]	0.005	-0.072	0.083	0.89
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.036	-0.100	0.028	0.27
- Late[5]	Reference	-	-	-

[1] 10 X Buctril-M® commercial herbicide formulation spray
[2] 1 X Buctril-M® commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

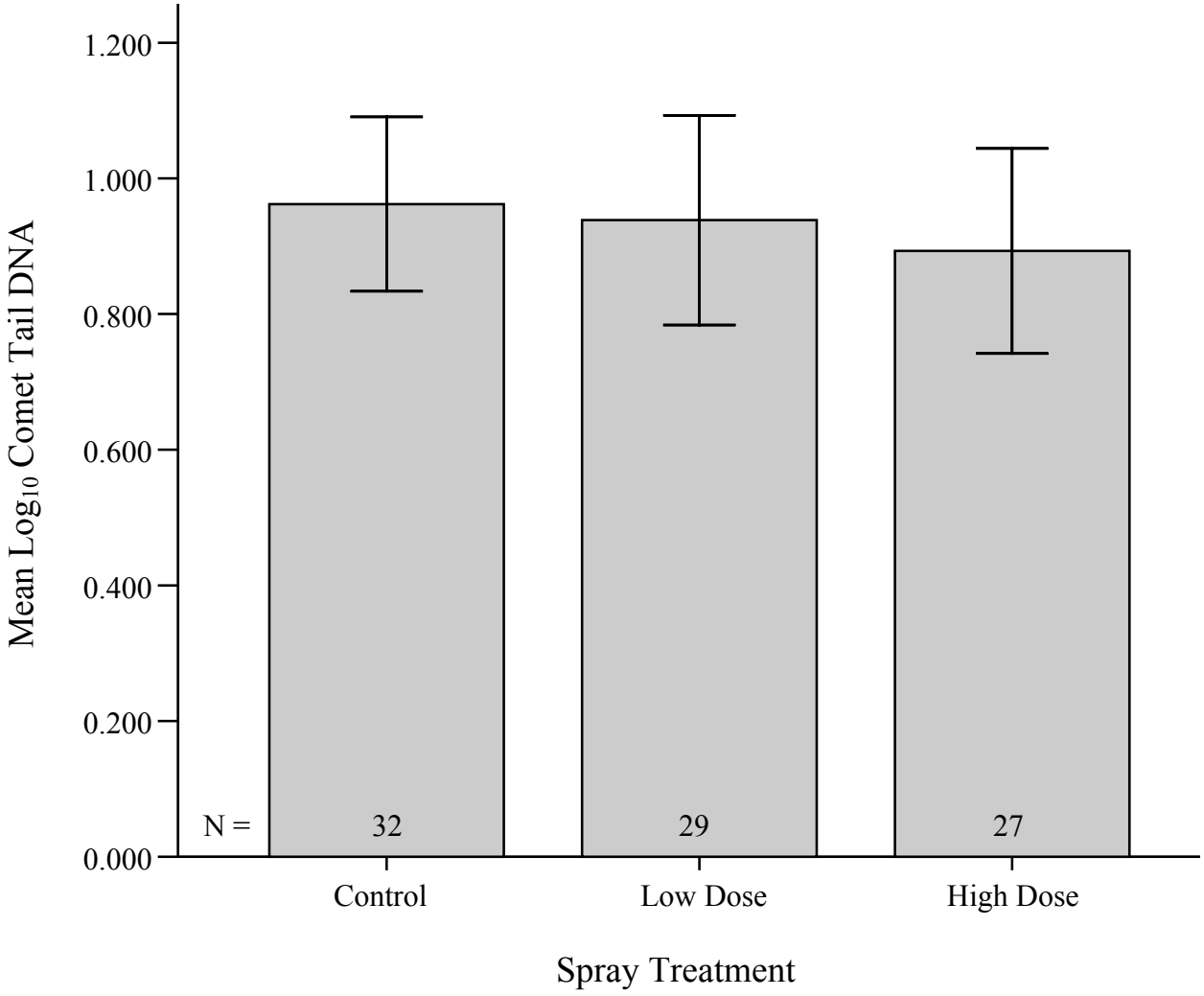

Figure 4-1. The simple association between comet tail DNA content of lymphocytes from blood of domestic chickens and *in ovo* Buctril-M® herbicide exposure. The bars represent mean log comet tail DNA (log_{10} transformed to attain normality) for the following groups: control (water), low dose (1 X Buctril-M®), and high dose (10 X Buctril-M®). Error bars represent ± SD.

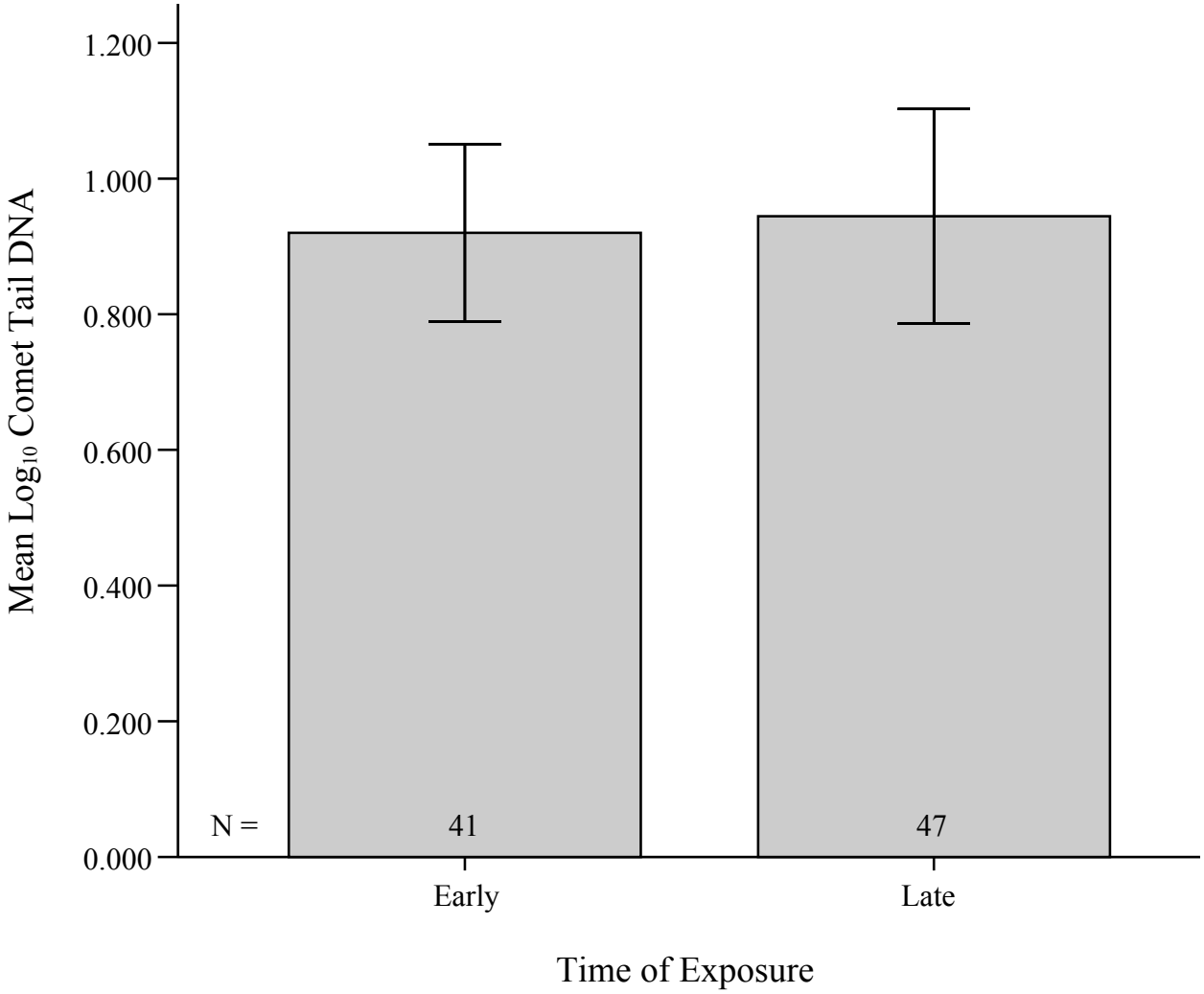

Figure 4-2. The simple association between comet tail DNA content of lymphocytes from blood of domestic chickens and the time of *in ovo* Buctril-M® herbicide spray application. The bars represent mean $\log_{10}$ comet tail DNA for the following exposure groups: early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD.

Table 4-5. Descriptive summary of flow cytometry outcome, half-peak coefficient of variation (HPCV, log_{10} transformed to attain normality) in DNA content of circulating erythrocytes from 21-day-old domestic chickens previously exposed *in ovo* to Buctril-M® formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	37	0.649	0.054	0.009	0.633	0.607	0.708
	Late[2]	29	0.621	0.054	0.010	0.623	0.580	0.663
1 X 2,4-D (Low Dose)	Early[1]	31	0.630	0.042	0.007	0.633	0.591	0.681
	Late[2]	36	0.630	0.054	0.009	0.613	0.591	0.672
10 X 2,4-D (High Dose)	Early[1]	39	0.643	0.055	0.009	0.633	0.602	0.681
	Late[2]	27	0.616	0.055	0.011	0.602	0.580	0.672

[1] Day 6 and [2] Day 15 of incubation

Table 4-6. Summary of univariate comparisons between the fixed effect factors time of exposure and herbicide treatment, and half-peak coefficient of variation (HPCV) in DNA content of erythrocytes from 21-day-old domestic chickens previously exposed *in ovo* to Buctril-M® formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	-0.011	-0.029	0.008	0.26
- Low Dose[2]	-0.006	-0.024	0.013	0.54
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.015	0.002	0.027	0.02*
- Late[5]	Reference	-	-	-

[1] 10 X Buctril-M® commercial herbicide formulation spray
[2] 1 X Buctril-M® commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

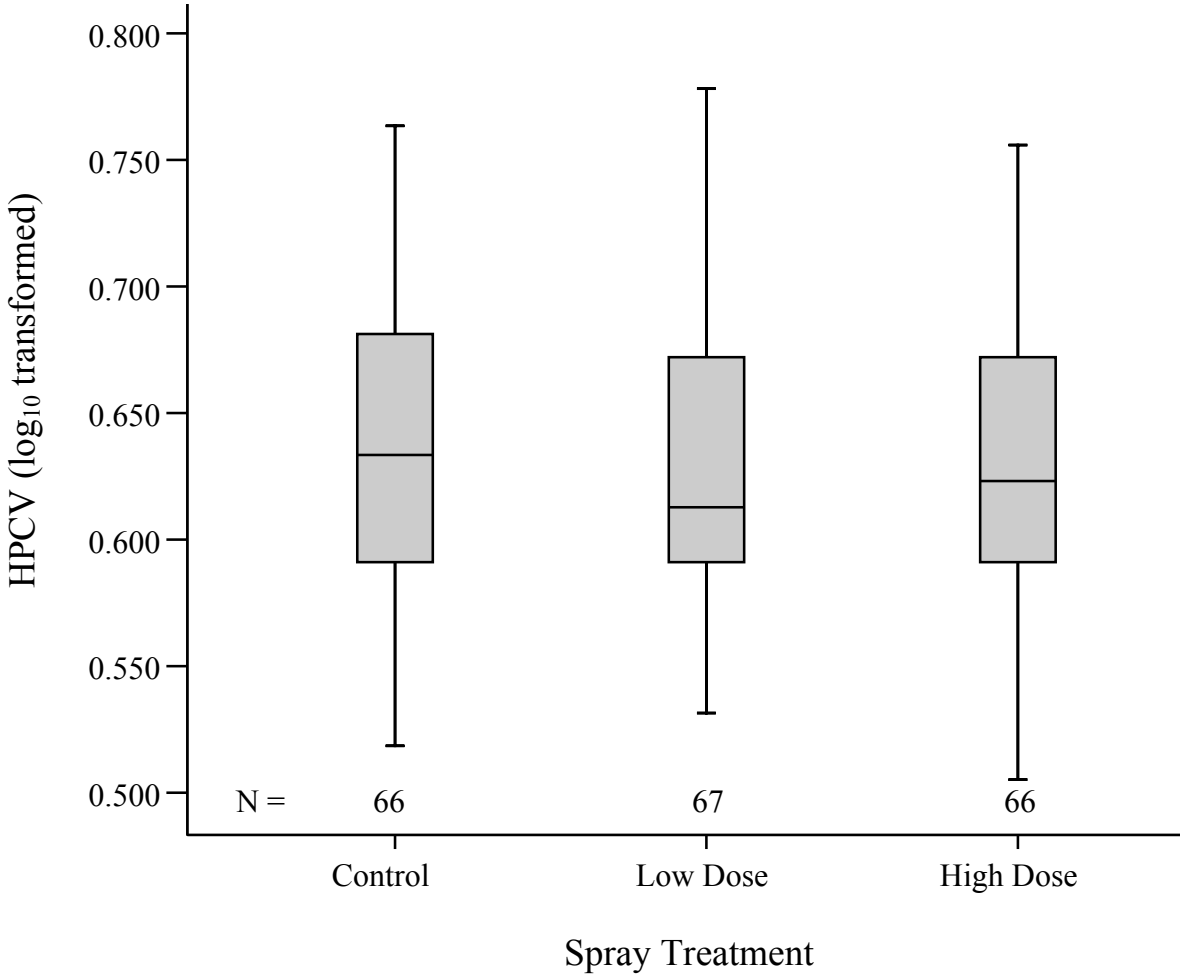

Figure 4-3. The simple association between the flow cytometry outcome half-peak coefficient of variation (HPCV, $\log_{10}$ transformed to attain normality) in DNA content of chicken erythrocytes and *in ovo* Buctril-M® herbicide exposure. Boxplots represent the following groups: control (water), low dose (1 X Buctril-M®), and high dose (10 X Buctril-M®). The center line represents the median, the box the interquartile range, the whiskers extend from the highest to lowest values excluding outliers.

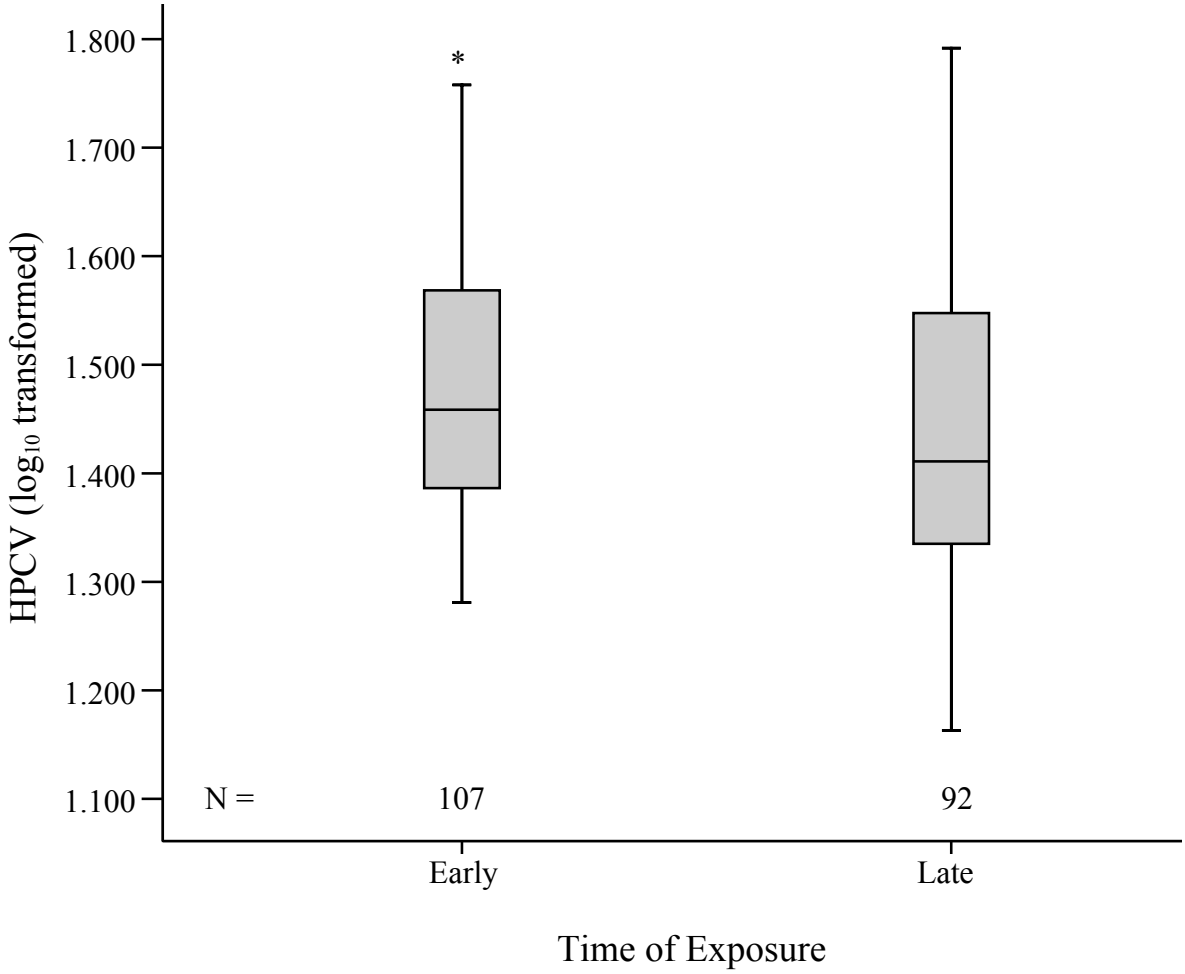

Figure 4-4. The simple association between the flow cytometry outcome half-peak coefficient of variation (HPCV, $\log_{10}$ transformed to attain normality) in DNA content of chicken erythrocytes and the time of *in ovo* Buctril-M® herbicide spray application. Boxplots represent the two times of herbicide exposure, early (day 6 of incubation) and late (day 15 of incubation). The center line represents the median, the box the interquartile range, the whiskers extend from the highest to lowest values excluding outliers. *Log_{10} HPCV values for the group exposed on day 6 were significantly different than those for the group exposed on day 15 ($P = 0.0210$).

4.4 Discussion

Newly hatched chickens exposed *in ovo* to the herbicide Buctril-M® demonstrated no evidence of genotoxic stress as measured by the comet assay. Differences in herbicide treatment (high and low concentrations) and times of exposure (early and late incubation stages) did not translate into noticeable effects on the structure of lymphocyte DNA in the final statistical model. Comet assay metrics percent DNA in the comet tail, tail moment, and tail length were all highly correlated. However, none of these endpoints to evaluate DNA strand breakage in isolated peripheral blood lymphocytes from herbicide treated chickens were affected by exposure.

In the flow cytometry assay, there were no treatment-related differences in mean values (measured by $\log_{10}$ HPCV) for variability in erythrocyte DNA content, and consequently no demonstrated association between clastogenic damage and herbicide concentration ($P = 0.53$). When exposure time was included in the final statistical model to assess the effects of spray exposure at different stages of embryonic development (either early or late incubation stages), a significant association was observed. HPCV values for birds from the early (embryonic day 6) *in ovo* spray treatment group were significantly higher ($P = 0.02$) than the values for birds exposed later in incubation (embryonic day 15). This association indicates that there is greater intercellular DNA content variability, and therefore increased clastogenic damage in bird embryos exposed to spray during the earlier developmental stage than in birds treated later in development. However, this effect is not related to Buctril-M® treatment, as there was no association with herbicide treatment in the final model. Therefore, the differences in DNA content variability observed cannot be explained as a toxicological effect of herbicide exposure.

Embryogenesis represents a critical developmental period in vertebrate species during which sensitivity to the toxic effects of agricultural and other environmental contaminants is often increased (Hoffman 1990b). In the present study, two distinct *in ovo* exposure timepoints were tested because the vulnerability of the avian embryo to toxic damage may change with the stage of embryonic development (DeWitt et al. 2005). In the spring, most agricultural areas of the Canadian prairies are sprayed with formulated herbicides to control for weed growth and optimize crop production. Spray applications may coincide with the nesting period of various species of ground nesting birds and waterfowl, therefore wild bird

eggs have the potential to be directly exposed to herbicide. Herbicide exposure may occur at any time after eggs are laid, so it was considered important to assess embryonic sensitivity to genetic damage during at least two distinct stages of development, in order to maximize the environmental applicability of the study.

In chickens, embryonic day 6 (E6) represents a relatively late stage of organogenesis, while embryonic day 15 (E15) coincides with a period of later differentiation (Patten 1971). By spraying eggs with Buctril-M® at these timepoints, embryos were exposed to herbicide during potentially sensitive stages of development. In chickens, the period from E0 up to E4 is the first important period for major organ formation and rapid tissue differentiation. However, toxicants can still have negative effects during later stages of avian development, so it is important to evaluate embryonic sensitivity throughout various stages (DeWitt et al. 2005).

Birds have long been used as monitors of environmental contamination and its effects on wild populations, or as animal models to evaluate the toxicological effects of commonly used agrochemicals (Hill and Hoffman 1984). For toxicity evaluation of pesticides and other environmental contaminants, *in ovo* exposure of bird embryos represents a useful approach to assess potential developmental effects. This study demonstrated that, in the chicken model, exposure to the commonly used herbicide Buctril-M® during different stages of development had no effect on the genetic integrity of newly hatched birds. Future studies of *in ovo* exposure to herbicides or other contaminants or stressors should focus on potential impacts during earlier stages of embryonic development, prior to day 6 of incubation.

CHAPTER 5

IMMUNOTOXIC EFFECTS OF *IN OVO* BUCTRIL-M® EXPOSURE IN DOMESTIC CHICKENS (*GALLUS GALLUS*)

Abstract

Conservation agriculture techniques such as reduced tillage and winter cereal seeding are commonly practiced on the Canadian prairies to reduce soil erosion and moisture loss. Many species of ground nesting birds, including upland game birds and waterfowl, are attracted to the increased vegetative cover provided by fall seeded crops and minimally tilled fields in the spring, and use this habitat for nesting. Since the nesting period for these species often coincides with herbicide treatment of many important cereal crops, eggs of ground nesting birds have the potential to be exposed during routine spray applications. The herbicide formulation Buctril-M®, containing a 50:50 mixture of bromoxynil (4-cyano-2,6-dibromophenol) and MCPA (4-chloro-2-methylphenoxyacetic acid), is commonly used on the Canadian prairies for weed control. Previous studies indicate the potential for these herbicide components to have sublethal effects on avian and mammalian species. However, information concerning possible effects of Buctril-M® exposure on the immune system of birds during embryonic development is inadequate to evaluate risk of contaminant exposure to eggs. This study investigated the effects of *in ovo* exposure to Buctril-M® on the immune system of newly hatched domestic chickens (*Gallus gallus*).

Fertile eggs were sprayed with Buctril-M® herbicide formulation at either normal field application rates or at 10 times recommended rates, on day 6 or 15 of incubation, to evaluate risks from herbicide exposure during early or late developmental stages, respectively. Control groups consisted of eggs sprayed with water only. The potential immunotoxic properties of Buctril-M® were assessed using standard assays to evaluate the general structure and function of the immune system, and specific immunomodulation effects on cell-mediated immunity and humoral immune function in newly hatched birds. The cell-mediated immune response was measured using a delayed-type hypersensitivity (DTH)

reaction to bovine serum albumin (BSA) in 21-day-old birds, and humoral immune function was assessed by stimulating systemic antibody production to BSA, as measured by ELISA. Additional tests of the immune system included differential white blood cell counts to determine heterophil/lymphocyte ratios, relative lymphoid organ weights and histopathology of immune organs.

The association between herbicide exposure and immunotoxicity was analyzed using a mixed linear statistical model. Herbicide treatment and time of exposure were accounted for as fixed factors. Results from the majority of immunoassays performed showed that *in ovo* exposure to the commercial herbicide mixture Buctril-M® at the application rates and incubation periods tested did not have a significant effect on the developing immune system in domestic chickens ($P > 0.05$). There was a significant association ($P = 0.0137$) between herbicide treatment and one of the general immune assessments - relative spleen weight; but no associations were observed between herbicide exposure and the functional assays.

5.1 Introduction

In recent years, the implementation of conservation farming techniques has improved soil quality and wildlife habitat on farms in the Canadian prairies. These sustainable practices include minimal soil tillage and the increased use of fall planted (winter cereal) crops, which reduce degradation of soil, water, and air quality, as well as maintain wildlife habitat and biodiversity. Implementation of these techniques generally results in increased ground cover in the spring. Therefore, farmland that receives minimal till (maintains vegetative crop cover) and/or is fall seeded (resulting in spring plant growth) is likely to represent better habitat for upland wildlife species than is found on conventionally-farmed land. However, land-use changes that favor wildlife production also generally require weed management practices which increase reliance on chemical weed control, and may increase the risk of herbicide exposure to nesting birds and their young.

The prairie provinces have the highest percentage of farmers in Canada who rely on routine pesticide use for insect and weed control (Boame 2005). Spring herbicide application is especially important for weed management on farmland receiving minimal till and in fields seeded with winter cereal crops, since it is the major alternative to tillage (Korol 2004). Conventional tillage methods effectively control the majority of persistent weed growth in cropland. However, with the adoption of conservation tillage practices (less mechanical

weed removal), cultivation operations are often replaced with intensified herbicide application to control weeds. Therefore, herbicide application rates are typically higher in low or no till fields compared with conventional fields (Gebhardt et al. 1985, Campbell 1999). As a commercial mixture, Buctril-M® is one of the top herbicides applied to winter cereal crops and fields receiving minimal till (Fowler 2002). Because typical spraying periods for spring weed control overlap with the nesting period of many species of ground-nesting waterfowl and upland game birds, the risk of egg exposure to the formulated herbicide product Buctril-M® is significant. The potential long-term effects of low rates of exposure to this herbicide in birds and other terrestrial wildlife remain to be determined, especially possible impacts of exposure during embryonic development.

In the past, studies investigating the toxicity of agricultural chemicals have mainly focused on the acute effects of single or large dose exposures. Recently, attention has been directed to evaluating the potential subtle effects of agrochemicals using more realistic environmental exposure situations. Pesticides are usually present in the environment at low to intermediate concentrations, which may not be overtly toxic to mature wildlife, but may have the potential to affect the immune systems of developing organisms.

The immature and early life stages of certain mammalian and non-mammalian species are likely the most vulnerable to immunotoxicity from contaminant exposure. Immune system dysfunction can result from alterations during development, and these effects may be long-term or not recognized as an adverse health effect until long after exposure to a potentially immunotoxic agent. Subtle effects of environmental contaminants on immune responses can be used as sensitive biomarkers of toxicant exposure (Keller et al. 2000, Grasman 2002). Numerous studies have been conducted in recent years to investigate the effects of environmental contaminants on immune function in domesticated and wild birds. The effects of metals, polychlorinated biphenyls (PCBs), pesticides, and organochlorine compounds have been evaluated in a variety of avian models, including chickens (*Gallus* spp.) (Knowles and Donaldson 1997, Lee et al. 2001, Lee et al. 2002, Finkelstein et al. 2003, Singhal et al. 2003), mallard ducks (*Anas platyrhynchos*) (Fairbrother and Fowles 1990, Fowles et al. 1997), avocets (*Recurvirostra americana*) (Fairbrother et al. 1994) western bluebirds (*Sialia mexicana*) (Fair and Myers 2002), American kestrels (*Falco sparverius*) (Smits and Bortolotti 2001), Japanese quail (*Coturnix japonica*) (Grasman and Scanlon

1995), gulls (*Larus* spp.) (Grasman et al. 1996, Bustnes et al. 2004), and Caspian terns (*Sterna caspia*) (Grasman et al. 1996, Grasman and Fox 2001). Several studies have assessed the effects of *in ovo* contaminant exposure in birds, and evaluated certain aspects of the immune response after introducing the contaminant at precise developmental stages (Bunn et al. 2000, Lee et al. 2001, Lee et al. 2002, Singhal et al. 2003). One study in particular (Dabbert et al. 1997) evaluated immunotoxic effects in Northern bobwhite (*Colinus virginianus*) chicks after *in ovo* exposure to the herbicide clopyralid (3,6-dichloro-2-pyridinecarboxylic acid) at field application rates.

Toxicological data concerning the potential effects of Buctril-M® exposure on wildlife, including avian and mammalian species, is limited. Much of the research that has been performed has involved determining the risk of Buctril-M® to aquatic invertebrates and fish when aquatic habitat is inadvertently contaminated (Buhl et al. 1993a, Buhl et al. 1993b, Morgan and Brunson 2002), or evaluating effects of the herbicide formulation using standard toxicity tests (e.g., LD_{50} = 368 mg/kg in the rat) with laboratory animal models (Bayer Crop Science). Although studies on terrestrial species are few, there has been some work on the relative toxicity of the individual herbicide components of Buctril-M®, bromoxynil (heptyl and octyl esters) and MCPA (2-ethyl hexyl ester), to mammalian and avian development.

Bromoxynil is a nitrile herbicide that is used for post-emergent control of broadleaf weeds through inhibition of photosynthesis. It is frequently tank mixed or commercially formulated with other herbicides such as 2,4-D and MCPA (Saskatchewan Agriculture and Food 2006). In acute toxicity tests bromoxynil has been shown to be highly to moderately toxic to many avian species including pheasants, hens, quail, and mallard ducks, with a dietary LC_{50} as low as 50 mg/kg (Kidd and James 1991). In chronic toxicity tests, it has been shown to affect bone development in rats and mice (evidence of supernumary ribs in both species), and is a suspected teratogen (Rogers et al. 1991, Chernoff et al. 1991). The sublethal toxic effects of bromoxynil are not as well known as other popular herbicides, and although bromoxynil has not been shown to be directly immunotoxic, the potential for this herbicide to have subtle effects on immune function as a result of its action as a developmental toxicant, appears reasonable.

The toxicity of chlorophenoxy herbicide compounds has been extensively studied. Since the late 1940s, this class of herbicides (acting as auxin hormone mimics in plants) has

seen continuous use in agriculture for the control of broadleaf weeds and woody plants. Along with 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxy-acetic acid (2,4,5-T), MCPA is one of the most common chlorophenoxyacetic acid herbicides. It is continuously used on the Canadian prairies as the main active herbicide ingredient in formulated sprays or as a popular component in herbicide mixtures with 2,4-D, dicamba, mecoprop, and bromoxynil (Saskatchewan Agriculture and Food 2006). Certain effects of MCPA exposure in avian embryos have been documented, but there is little information about the action of the herbicide on the immune system in developing birds.

Numerous studies have evaluated the toxicity of chlorophenoxyacetic acid compounds. However, the potential for chemicals in this herbicide class to have subtle effects on certain physiological functions is still being debated. Several studies have investigated the immunotoxic effects of the related chlorophenoxy herbicide 2,4-D, while only a few studies have evaluated the effects of MCPA on the immune system. Immune effects of 2,4-D in laboratory animals include immunosuppression (Blakley 1997). Farmers exposed to 2,4-D and MCPA through regular agricultural use, exhibit short term immunosuppressive effects (Faustini 1996). Evidence suggesting that 2,4-D could potentially have long-term effects on the immune response raises questions about the immunotoxic nature of MCPA, because these chemicals are structurally and functionally similar. Studies with laboratory animals have shown that other chemicals related to MCPA have the potential to be immunotoxic or alter immune function. However, little is known about the subtle effects of MCPA on the immune function of wild species, especially when exposure occurs during embryonic development. This study was intended to investigate whether *in ovo* exposure to bromoxynil and MCPA in the formulated herbicide product Buctril-M® was associated with immunomodulation in domestic chickens (*Gallus gallus*), chosen as surrogates for upland game birds. The potential immunotoxic properties of Buctril-M® were assessed using standard assays to evaluate the general function of the immune system, and specific tests to identify potential changes to cell-mediated immunity and humoral immune function in hatchlings.

The DTH test has been used successfully to assess modulation of cell-mediated immune function in birds exposed to environmental contaminants. In mallards (*Anas platyrhynchos*), a relationship (approaching statistical significance) was found between a

decreasing T cell inflammatory response in the DTH test and increasing selenomethionine dose (Fairbrother and Fowles 1990). *In ovo* exposure to lead was associated with depressed DTH response in chickens (*Gallus gallus*) (Lee et al. 2001, Lee et al. 2002). In the present study, the cell-mediated immune response in 21-day-old birds exposed *in ovo* to Buctril-M® was evaluated using a DTH reaction against bovine serum albumin (BSA).

A balanced evaluation of immune function requires the assessment of the second arm of the acquired (specific) immune response, humoral immunity. One of the most powerful tests to evaluate B cell mediated immune function is to measure the strength of the humoral (antibody-mediated) immune response following antigen exposure. The ability of an individual to produce antigen-specific antibodies can be measured using the enzyme-linked immunosorbent assay (ELISA) (Smits and Janz 2005). Several ecotoxicological studies have used the ELISA technique to detect specific immunoglobulin levels in wild birds following antigen exposure to stimulate specific antibody response. Bustnes et al. (2004) found that female glaucous gulls (*Larus hyperboreus*) with high blood concentrations of organochlorine pesticides showed a decreased immune response to novel antigen immunization. The ELISA has also been used to measure antibody responses in western bluebird (*Sialia mexicana*) nestlings exposed to lead shot (Fair and Myers 2002) and in American kestrels (*Falco sparverius*) exposed to polychlorinated biphenyls (Smits and Bortolotti 2001). In studies with domestic chickens, the ELISA has been used to measure antigen-specific immunoglobulin levels in birds exposed *in ovo* to a pesticide (Singhal et al. 2003) and lead (Lee et al. 2002). In the present study, the primary and secondary humoral immune response in chicks exposed *in ovo* to Buctril-M® spray was measured after sensitizing birds with BSA at 7- and boosting at 14- days of age, respectively.

Another accepted method of assessing the effect of xenobiotics on the immune system is to examine the characteristics of associated cells, tissues, and organs in exposed animals. Sampling and studying these components, termed immunopathology, provides general information about immune structure and function (Keller et al. 2000). The present investigation attempted to evaluate the overall health of the immune system and associated components using various tests as tools to assess the potential immunotoxicity of Buctril-M® (Schuurman et al. 1994). Selected tests including differential white blood cell counts to

measure potential lymphocyte changes, and examination of immune organ weights and histopathology to assess immune organ health.

The *in ovo* herbicide exposure design employed was intended to simulate a scenario in which eggs of upland game birds are sprayed with herbicide during weed control operations. The results of this study will help to determine the subtle impacts of Buctril-M® on immune health following exposure during different stages of avian embryonic development, using domestic chicks as the animal model.

5.2 Materials and Methods

5.2.1 Animal Model

Fertile chicken (*Gallus gallus*) eggs from a White Leghorn/Brown Leghorn cross were incubated at 37.5°C and approximately 80% humidity in circulated air incubators (Hova-Bator®, G.Q.F. Manufacturing Co., Savannah, Georgia, USA) until 1 day post-hatch (about day 23). Automatic egg turners (Hova-Bator®, G.Q.F. Manufacturing Co., Savannah, Georgia, USA) were used for the first 18 days of incubation. On day 19 of incubation, egg turners were removed and eggs were placed on the wire floor of the incubator. Humidity was increased to approximately 90% in accordance with hatching requirements. Chicks were transferred to heated brooders with raised wire floors at one to two day(s) of age, and maintained on *ad libitum* chick starter and fresh water for the duration of the study.

5.2.2 Herbicide Spray Exposures

Fertile chicken eggs (4 replicate groups each of 120 eggs) were randomly assigned to one of six incubators (20 per incubator), and each incubator was randomly assigned to a specific treatment. In avian embryos, different types of developmental effects may be attributed to immunomodulation during critical periods of embryonic development. In order to account for time-specific vulnerability of the embryos, eggs were exposed to the herbicide during either an early (day 6) or late (day 15) stage of incubation (DeWitt et al. 2005). Eggs were sprayed with the commercial Buctril-M® formulation (Bayer CropScience Inc., Calgary, Alberta, Canada) at one of two different concentrations: 1) Low dose groups (early and late) were sprayed with Buctril-M® at the recommended field application rate for winter wheat (0.55 L ai ha^{-1}) (SAFRR 2005). This was equivalent to 4.94 ml of formulated Buctril-M® per litre of spray solution. 2) High dose groups (early and late) were sprayed with 49.4 ml of formulated Buctril-M® per litre, equivalent to 10 times the recommended concentration

of herbicide, to simulate a worst-case exposure. Additional groups of eggs (early and late) were sprayed at the same time points with water only to act as negative controls. The spray treatments were applied using an agricultural field spray simulator (Figure 2-1) at the Agriculture and Agri-Foods Canada Research Centre in Saskatoon, Saskatchewan, to reproduce actual field application conditions as closely as possible. Eggs were masked prior to spraying to ensure that every egg received equal amounts of herbicide (Figure 2-1). The six treatment groups comprising each replicate were: high Buctril-M® dose (early and late incubation exposures); low Buctril-M® dose (early and late); and negative control (water only, early and late). The four replicate experiments were spaced about two weeks apart.

5.2.2.1 Quantifying Herbicide Exposure

Herbicide deposition on the surface of the eggs was quantified by spraying surplus eggs with fluorescein dye solution (10% (w/w) fluorescein sodium salt in water) at the same application rate as the herbicide treatments. The amount of fluorescein dye deposited on the exposed portion of the eggs was determined by rinsing the eggs to remove the dye, and determining the amount of fluorescein in the rinsate. The rinsate fluorescence was measured at 498 nm using a spectrofluorometer (Shimadzu RF-1501, Shimadzu Corporation, Columbia, MD, USA).

5.2.3 Sample Collection

Samples for immunoassays were collected at four different time points post hatching. Blood was collected from all 7-day-old birds to determine baseline serum antibody titres against BSA, and birds were immunized (primary exposure) with BSA. On day 14, blood was collected to determine the primary antibody response to the BSA immunization, and birds were immunized again with BSA (secondary exposure). On day 20, pre-exposure wing web measurements were taken, followed by intradermal wing web injections with BSA for the DTH test. On day 21, post-exposure wing web measurements were taken, and blood was collected to determine the secondary antibody response to the BSA immunization on day 14, and for differential white blood cell counts. Birds were subsequently euthanized, and selected immune organs (thymus, spleen, bursa of Fabricius) were collected, weighed and preserved for histopathological examination.

The ELISA was performed on blood serum samples collected from 7-day-old (baseline antibody titres), 14-day-old (primary antibody titres), and 21-day-old (secondary

antibody titres) birds previously immunized with BSA. Birds were sensitized on day 7 and boostered on day 14 by injecting 0.5 ml of BSA at 4 mg/ml in sterile physiological saline subcutaneously into the dorsal scapular region. Blood samples were collected from the jugular vein with a heparinized syringe into Eppendorf® microcentrifuge tubes, and kept on ice until further sample processing was performed. Within 6 hours of blood collection, samples were centrifuged for 10 min at 5000 rpm (Eppendorf Centrifuge 5415D, Brinkmann Instruments Inc., Westbury, NY, USA). Blood serum was carefully withdrawn from the tubes with a pipette, transferred into low temperature freezer vials, and stored at -80°C until the ELISA was performed. Unless otherwise indicated, all chemicals and reagents were obtained from Sigma Aldrich Canada Ltd. (Oakville, ON), and laboratory supplies and disposables were purchased from VWR International (Mississauga, ON).

5.2.4 ELISA Protocol for Detection of Specific IgG Antibodies

Humoral immune function was evaluated by stimulating antibody production in chicks. The response was measured using a modified ELISA technique (Smits and Bortolotti 2001) to measure IgG class antibody titres in the blood serum of birds sensitized with BSA. Microtiter plates (96-well, flat bottom, Nunc-brand, Nalgene) were coated with 100 µl/well BSA at 0.5 µg/ml in carbonate coating buffer (pH 9.6) by incubating at 4˚C for 15 hours. Following incubation, plates were rinsed 4X with 0.05% phosphate buffered saline-Tween 20 (PBS-T, pH 7.3), and residual binding sites blocked with 5% dried skim milk (100 µl/well) for one hour at room temperature. Plates were rinsed 4X with PBS-T after blocking. Serum samples and standards were diluted in PBS-T. Positive and negative controls consisted of pooled serum from day 21 and day 7 (pre-sensitized) birds, respectively. Twofold dilutions of sera (100 µl/well), beginning with a dilution of 1/50, were added to duplicate rows across the plates, followed by incubation at room temperature for two hours. Plates were then rinsed 4X with PBS-T and 100 µl of rabbit anti-chicken IgG (1:400, Bethyl Laboratories, Inc., Montgomery, TX, USA) was added to each well, followed by incubation at room temperature for one hour. Plates were again rinsed 4X with PBS-T, and 100 µl of goat anti-rabbit (1:800, Bethyl Laboratories, Inc., Montgomery, TX, USA) horseradish peroxidase conjugate was added to each well, followed by incubation at room temperature for 1 hour. Plates were again rinsed 4X with PBS-T, and 100 µl of ABTS® horseradish peroxidase substrate (2,2'-azino-di (3-ethyl-benzthiazoline-6-sulfonate) in glycine/citric acid buffer,

KPL, Gaithersburg, MD, USA) was added to all wells, and the plates were incubated in the dark at room temperature for 12 minutes. Finally, 100 µl of stop solution (1% SDS) was added to all wells, and absorbance at 405 nm was measured using a microplate spectrophotometer (SPECTRAmax® 190, Molecular Devices Corporation, Sunnyvale, CA, USA) using SoftMax Pro Software, version (SOFTmax® PRO, version 4.0, Molecular Devices Corporation, Sunnyvale, CA, USA). Anti-BSA antibody titres for chicks exposed *in ovo* to Buctril-M® are the reciprocal of the highest dilution of serum with an optical density value greater than the cutoff value. The cutoff value was the mean optical density value for the pooled negative control serum sample (containing baseline antibody levels). Statistical analysis was performed on the antibody titre (log transformed to attain normality) for samples collected from 14- and 21-day-old birds. These values reflect the strength of the primary and secondary humoral response, respectively.

5.2.5 Delayed-type hypersensitivity (DTH) Test for T cell Response

Immunization with BSA for assessment of humoral immune function also acted as antigen sensitization for the DTH test. This test was conducted on 20-day-old chicks. The right wing web of each bird was plucked free of feathers, marked to identify the injection site, and the thickness of the marked area was measured to the nearest 0.01 mm using a spring-loaded dial micrometer (Mitutoyo, Precision Graphic Instruments, Spokane, WA, USA). Three measurements were taken of the same site, and the mean value was recorded. The marked site was swabbed with alcohol (70% ethanol) and injected intradermally with 0.1 ml of BSA (20 mg/ml in saline) using a 27-g needle. The thickness of the injection site was re-measured 24 hrs later by the same operator using the same technique, and the DTH response was reported as the difference in wing web thickness (Smits et al. 1999) using the following formula:

$$\frac{\text{Mean thickness of wing web (post-injection)} - \text{Mean thickness of wing web (pre-injection)}}{\text{Mean thickness of wing web (post-injection)}}$$

5.2.6 Hematology – Differential Leukocyte Count

Blood smears (two per bird) prepared from samples collected on day 21 were air dried and stained using Diff-Quik® (Dade Behring Inc., Newark, DL, USA). The ratio of heterophils to lymphocytes in peripheral circulation was determined for each bird by counting 100 leukocytes per slide at 400X total magnification. The ratio of heterophils to

lymphocytes is a useful indication of stress in some avian species (Gross and Siegel 1983, Grasman et al. 1996, Maxwell and Robertson 1998).

5.2.7 Relative Organ Weights and Histopathology

On day 21, birds were weighed (± 0.01g), blood sampled, and euthanized by cervical dislocation. Selected lymphoid organs (thymus, spleen, and bursa of Fabricius) were collected and fixed in 10% neutral buffered formalin within 15 min of death. Prior to fixation, the spleen and bursa of Fabricius were trimmed of adherent fat and connective tissue, and the mass of each organ determined (± 0.01g) in order to evaluate the relative organ weight, or somatic index (somatic index = organ weight/(body weight – organ weight)).

For histopathological examination, cross-sections of two thymic lobes, and the spleen and bursa of Fabricius were embedded in paraffin, routinely processed, stained with hematoxylin and eosin, and examined by a veterinary pathologist blind to the treatment groups. Cross-sections of all three organs were examined for evidence of overt pathology, including depleted lymphocytes or lymphoid atrophy. The organs were also evaluated on the basis of individual organ criteria, and subjectively compared between control and treatment groups. In the thymus, the relative thickness of the cortex and medulla was compared. In the spleen, the relative proportion of the white and red matter was evaluated. In the bursa of Fabricius, the size of the lymphoid follicles and the follicular cortico-medullary ratio were compared between control and treatment groups. Rates of mitoses and apoptosis in the bursa and thymus were also compared between treatments as a subjective measure of cellular normality and function.

5.2.8 Statistical Analysis

Descriptive statistics for all outcomes were compiled using SPSS (v.14.0, SPSS Inc., Chicago, IL, USA) and SYSTAT (v.11.0, SPSS Inc., Chicago, IL, USA). Summaries are reported for the subset of animals used for the ELISA (N=196), the DTH test (N=104), the relative immune organ weight/body weight ratios (N=183), and the assessment of heterophil/lymphocyte ratios (N=199). The normal distribution of all assay variables was assessed using the Shapiro-Wilk test for normality. All parameters were $\log_{10}$ transformed to attain normality if the p-value given by this test was low (i.e. < 0.5).

The association between herbicide exposure and immune endpoints was analyzed using a mixed linear model (PROC MIXED in SAS v.8.2 for Windows, SAS Institute, Cary, NC, USA). Incubator and experimental group were included as random effects to account for clustering of the observations as a result of separate incubator designations and replicated experiments, respectively. First, time of exposure (early or late incubation stage) and then herbicide treatment level (high or low concentration or water control) were assessed as fixed effect factors in a model including the random effects. Where time of exposure and herbicide treatment were both potentially important factors ($P < 0.25$), both were included in a model to assess confounding effects. Variables were retained in the final model if they were significant ($P < 0.05$), or acted as important confounders (i.e. adjustment for the variable changed the other coefficient by more than 10%). If both time of exposure and herbicide treatment were significant ($P < 0.05$), the model was then tested for interaction.

5.3 Results

Herbicide deposition on the surface of the eggs was quantified in a separate study in which fluorescein dye was sprayed onto masked eggs, eggs were rinsed, and the amount of fluorescein in the rinsate was measured using a spectrofluorometer. Mean doses of the Buctril-M® herbicide deposited onto masked eggs were calculated for low and high dose exposure groups, and reported in Table 4-1.

Descriptive statistics for the measurements of serum antibody concentrations in chickens following BSA immunization are summarized in Table 5-1. Serum samples from both 14-day-old chicks (post-BSA primary immunization), and 21-day-old chicks (post-BSA secondary immunization) contained higher concentrations of anti-BSA antibodies (positive reaction against BSA at a higher dilution) than pre-immunization sera, indicating that birds responded as expected to BSA immunization. Antibody concentrations were broadly similar to controls and Buctril-M® treated birds (for both times of exposure) suggesting that neither herbicide treatment nor timing of spray exposure affected the humoral immune response of these birds.

Table 5-2 summarizes the univariate comparisons between the fixed effect factors, herbicide treatment and time of exposure, and antibody production as measured by ELISA. For the 14-day-old chicks, neither herbicide treatment ($P = 0.75$) or time of exposure ($P = 0.81$) were not considered to be important factors in the initial model ($P > 0.25$). For 21-day-

old chicks, herbicide treatment (P = 0.63) and time of exposure (P = 0.61) were not considered potentially important factors in influencing antibody response ($P > 0.25$). Figures 5-1 and 5-2 show the relationships between all mean sera samples and the fixed effect factors herbicide treatment and time of exposure, respectively.

Descriptive statistics for the outcomes from the DTH test are summarized in Table 5-3. The DTH response was assessed by measuring the change in thickness of the wing web after intradermal injection of BSA. Simple comparisons of the test outcomes from the herbicide treated birds to those of the control groups revealed no differences. Table 5-4 summarizes the univariate comparisons between the fixed effect factors herbicide treatment and time of spray exposure, and the mean differences in wing web thickness. Herbicide treatment (P = 0.65) and time of exposure (P = 0.65) were not associated with the ability of birds to mount a DTH response. The relationships between the mean differences in wing web induration and the fixed effect factors, herbicide treatment and time of exposure, are shown in Figures 5.3 and 5.4, respectively.

Descriptive statistics for the ratio of heterophils to lymphocytes in peripheral blood samples from 21-day-old chicks are summarized in Table 5-5. Table 5-6 summarizes the univariate comparisons between the mean log ratios and both fixed effect factors. Herbicide treatment (P = 0.99) and time of exposure (P = 0.27) and did not have a significant effect on the numbers of heterophils and lymphocytes in whole blood samples. Figures 5-5 and 5-6 demonstrate that neither of the fixed effect factors had a significant effect on heterophil/lymphocyte ratios in chickens.

Descriptive statistics for the relative spleen weight/body weight ratios in 21-day-old chickens are summarized in Table 5-7. Only herbicide treatment was found to be a significant factor in the initial analysis ((Table 5-8, P = 0.01), influencing differences between groups in the final univariate comparison model. Graphs showing the association between relative spleen weight and both fixed factors are presented in Figures 5-7 and 5-8. A summary of the descriptive statistics for the relative bursa of Fabricius weight/body weight ratios are presented in Table 5-9. Neither herbicide treatment (P = 0.11) or exposure time (P = 0.71) contributed to differences in relative bursal weight between groups (as shown in Table 5-10, Figure 5-9 and 5-10).

Histological examination of lymphoid organs (cross-sections of thymus, spleen and bursa of Fabricius) from birds treated *in ovo* with high dose Buctril-M® formulation spray and water (control) was performed by a wildlife pathologist. All three tissues were examined for overt pathological features, particularly lymphocyte depletion or lymphoid atrophy. In all tissues examined, from both high dose herbicide and water treatment groups no evidence of treatment-induced pathology was reported, and there was no indication that the lymphoid organs from either group were different histologically.

Table 5-1. Descriptive summaries of the serum dilutions (reciprocal values, $\log_{10}$ transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA. Serum was collected from 14- (a) and 21-day-old chicks (b), exposed *in ovo* to Buctril-M® spray and immunized with BSA at 7 and 14 days of age, to evaluate the effects of exposure on the primary and secondary antibody responses, respectively.

(a) **Serum from 14-day-old chicks**

							Percentiles	
Treatment	**Exposure Time**	**N**	**Mean**	**SD**	**SE**	**Median**	**25th**	**75th**
Water (Negative Control)	Early[1]	35	2.843	0.478	0.081	2.903	2.301	3.204
	Late[2]	28	2.839	0.647	0.122	2.903	2.075	3.505
1 X Buctril-M® (Low Dose)	Early[1]	31	2.631	0.529	0.095	2.602	2.301	3.204
	Late[2]	36	2.953	0.560	0.093	3.204	2.602	3.430
10 X Buctril-M® (High Dose)	Early[1]	39	2.988	0.458	0.073	2.903	2.602	3.204
	Late[2]	27	2.736	0.510	0.098	2.602	2.301	3.204

(b) **Serum from 21-day-old chicks**

							Percentiles	
Treatment	**Exposure Time**	**N**	**Mean**	**SD**	**SE**	**Median**	**25th**	**75th**
Water (Negative Control)	Early[1]	35	3.041	0.599	0.101	2.903	2.602	3.505
	Late[2]	28	2.774	0.604	0.114	2.602	2.301	3.129
1 X Buctril-M® (Low Dose)	Early[1]	31	2.825	0.538	0.097	2.602	2.602	3.204
	Late[2]	36	3.162	0.632	0.105	3.204	2.602	3.806
10 X Buctril-M® (High Dose)	Early[1]	39	3.196	0.616	0.099	3.204	2.602	3.804
	Late[2]	27	2.903	0.578	0.111	2.903	2.301	2.903

[1] Day 6 and [2] Day 15 of incubation

Table 5-2. Summary of univariate comparisons between the fixed effect factors, herbicide treatment and time of exposure, and the serum dilutions (reciprocal values, $\log_{10}$ transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA. Statistical comparisons are summarized for the serum samples collected from 14- (a) and 21-day-old chicks (b) exposed *in ovo* to Buctril-M® and immunized with BSA at 7 and 14 days of age.

(a) **Serum from 14-day-old chicks**	**Regression coefficient (β)**	**95% Confidence Intervals for β**		***P* value**
		Lower	**Upper**	
Herbicide Treatment				
- High Dose[1]	0.0195	-0.2470	0.2860	0.89
- Low[2]	-0.0769	-0.3373	0.1835	0.56
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.0257	-0.2351	0.1837	0.81
- Late[5]	Reference	-	-	-
(b) **Serum from 21-day-old chicks**				
Herbicide Treatment				
- High Dose[1]	0.1597	-0.1698	0.4891	0.34
- Low Dose[2]	0.0627	-0.2581	0.3835	0.70
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.0670	-0.1918	0.3257	0.61
- Late[5]	Reference	-	-	-

[1] 10 X Buctril-M® commercial herbicide formulation spray
[2] 1 X Buctril-M® commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

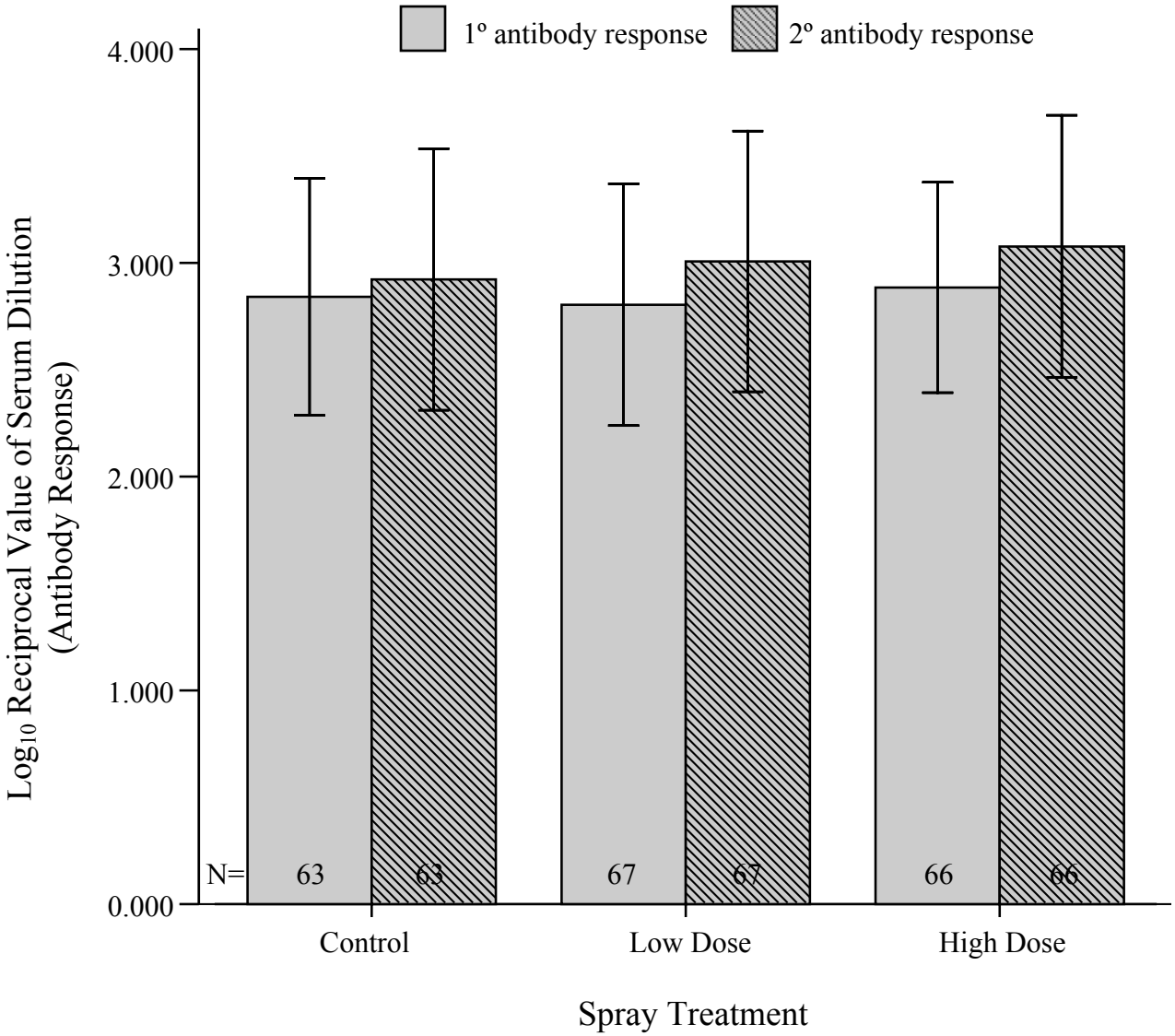

Figure 5-1. The simple association between the reciprocal of the highest dilution of serum (log transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA and *in ovo* Buctril-M® herbicide exposure in chickens. The bars represent the mean reciprocal values of serum dilutions for samples collected from 14- and 21-day-old birds, and therefore reflect the strength of the primary and secondary response, respectively. Bars are grouped into the following treatments: control (water), low dose (1 X Buctril-M®), and high dose (10 X Buctril-M®). Error bars represent ± SD of the mean.

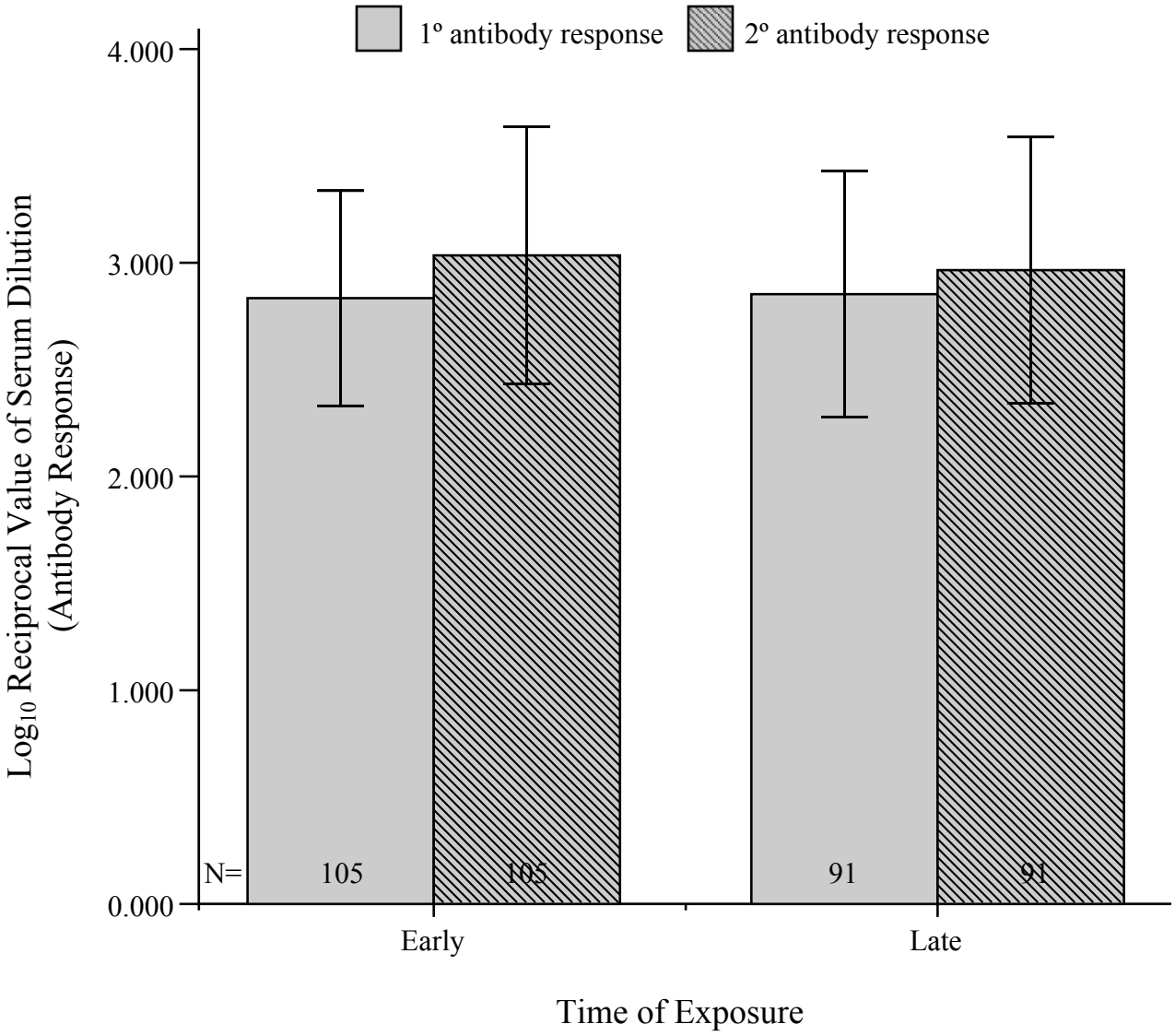

Figure 5-2. The simple association between the reciprocal of the highest dilution of serum (log_{10} transformed to attain normality) that demonstrated a positive reaction against BSA in the ELISA and the time of *in ovo* Buctril-M® herbicide spray application in chickens. The bars represent the mean reciprocal values of serum dilutions for samples collected from 14 and 21-day-old birds, and therefore reflect the strength of the primary and secondary humoral response, respectively. Bars are grouped into the following exposure groups: early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

Table 5-3. Descriptive summary of the DTH response, as measured by differences in wing web thickness (mm) following intradermal BSA injection, in 21-day-old domestic chickens exposed *in ovo* to Buctril-M® formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	18	0.14	0.14	0.03	0.17	0.05	0.23
	Late[2]	20	0.12	0.17	0.04	0.10	0.07	0.19
1 X Buctril-M® (Low Dose)	Early[1]	16	0.11	0.11	0.03	0.12	0.02	0.21
	Late[2]	19	0.18	0.16	0.04	0.19	0.05	0.32
10 X Buctril-M® (High Dose)	Early[1]	15	0.17	0.06	0.02	0.16	0.12	0.22
	Late[2]	16	0.15	0.08	0.02	0.13	0.09	0.20

[1] Day 6 and [2] Day 15 of incubation

Table 5-4. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the wing web DTH response to BSA injection, measured in 21-day-old domestic chickens exposed *in ovo* to Buctril-M® formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	0.03	-0.04	0.09	0.38
- Low Dose[2]	0.02	-0.04	0.08	0.50
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.01	-0.06	0.04	0.65
- Late[5]	Reference	-	-	-

[1] 10 X Buctril-M® commercial herbicide formulation spray
[2] 1 X Buctril-M® commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

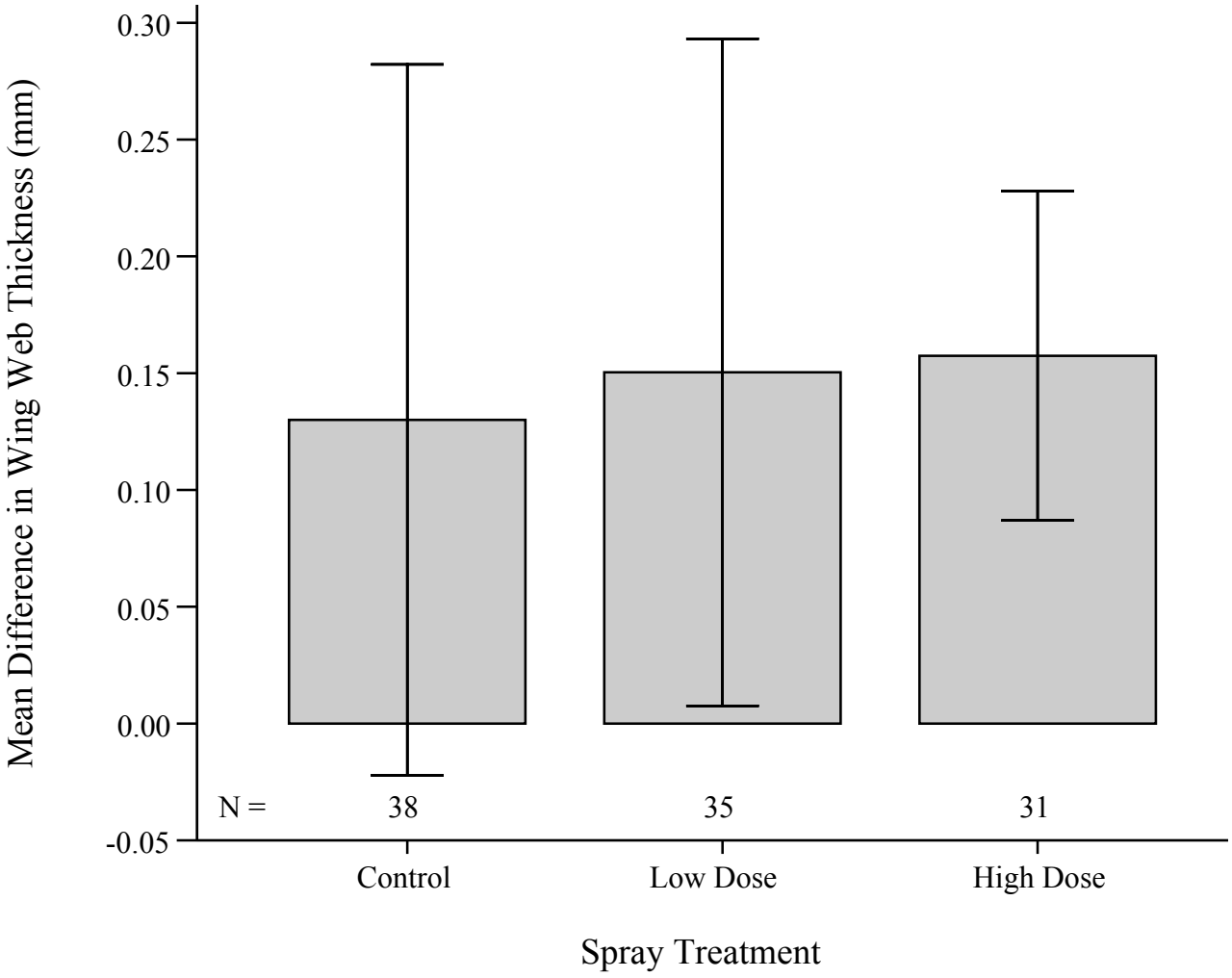

Figure 5-3. The simple association between DTH response (increase in wing web thickness, mm) in 21-day-old domestic chickens and *in ovo* Buctril-M® herbicide exposure. The bars represent mean DTH response for the following groups: control (water), low dose (1 X Buctril-M®), and high dose (10 X Buctril-M®). Error bars represent ± SD of the mean.

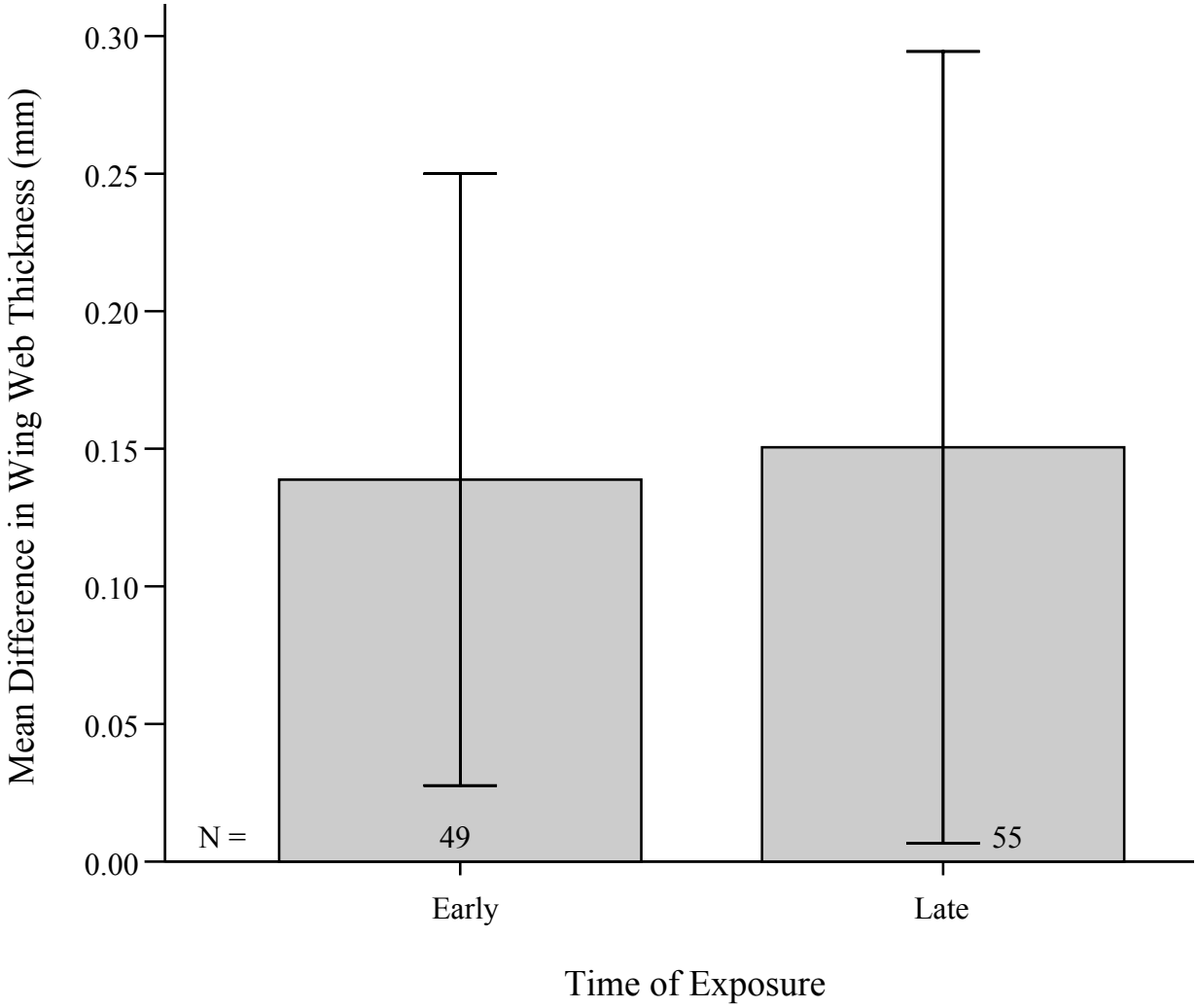

Figure 5-4. The simple association between DTH response (increase in wing web thickness, mm) in 21-day-old domestic chickens and the time of *in ovo* Buctril-M® herbicide spray application. The bars represent mean DTH response for the following exposure groups: early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

Table 5-5. Descriptive summary of the heterophil/lymphocyte ratios in peripheral blood from 21-day-old domestic chickens exposed *in ovo* to Buctril-M® formulation spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	37	0.527	0.261	0.043	0.483	0.331	0.721
	Late[2]	29	0.629	0.327	0.061	0.517	0.442	0.693
1 X Buctril-M® (Low Dose)	Early[1]	31	0.526	0.213	0.038	0.509	0.354	0.661
	Late[2]	36	0.623	0.915	0.152	0.454	0.297	0.606
10 X Buctril-M® (High Dose)	Early[1]	39	0.575	0.224	0.036	0.550	0.409	0.679
	Late[2]	27	0.579	0.226	0.044	0.525	0.409	0.750

[1] Day 6 and [2] Day 15 of incubation

Table 5-6. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the heterophil/lymphocyte ratios in peripheral blood from 21-day-old domestic chickens exposed *in ovo* to Buctril-M® formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	-0.008	-0.164	0.147	0.92
- Low Dose[2]	0.002	-0.153	0.157	0.98
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.070	-0.197	0.056	0.27
- Late[5]	Reference	-	-	-

[1] 10 X Buctril-M® commercial herbicide formulation spray
[2] 1 X Buctril-M® commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

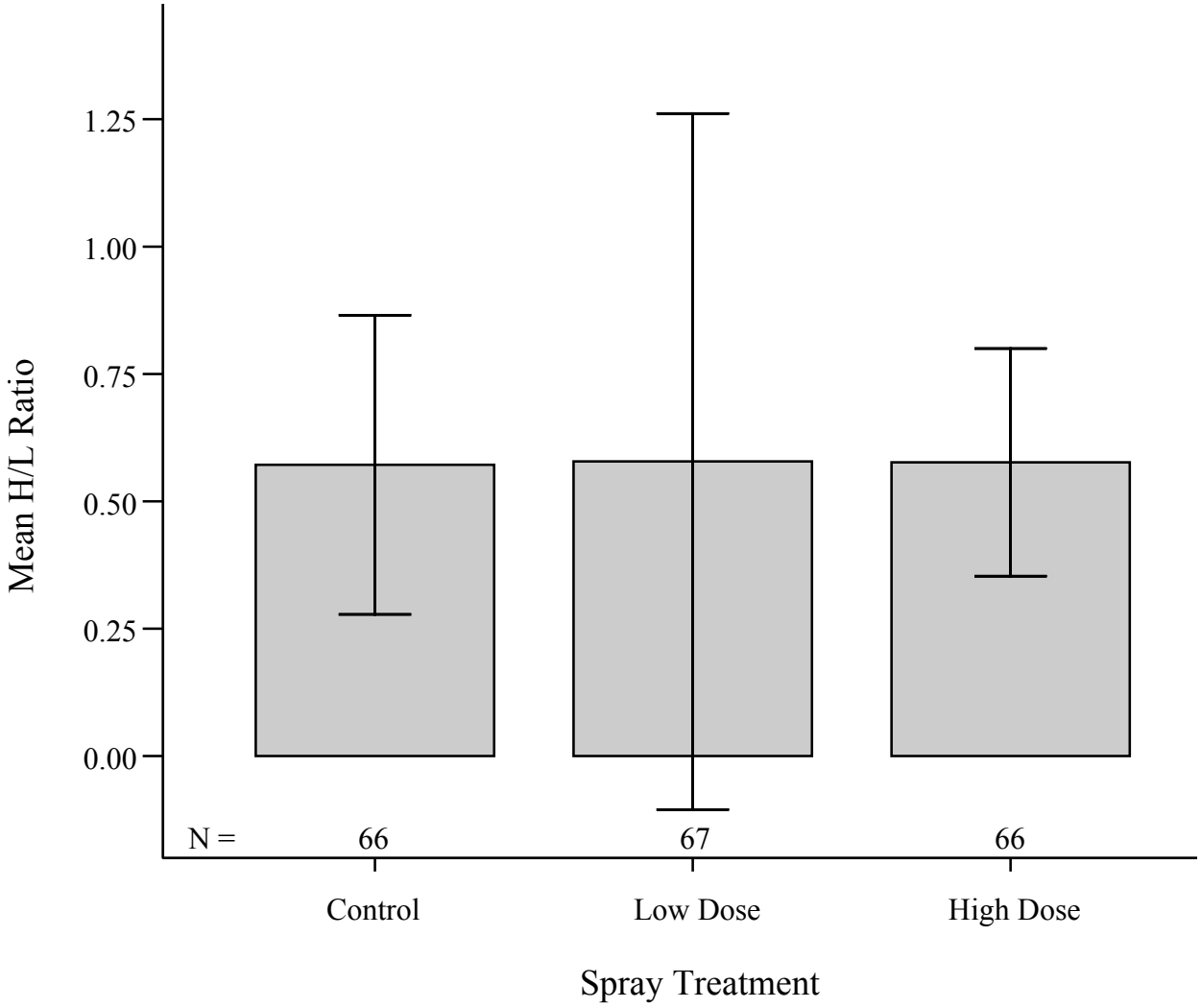

Figure 5-5. The simple association between heterophil/lymphocyte (H/L) ratios in peripheral blood from 21-day-old domestic chickens and *in ovo* Buctril-M® herbicide exposure. The bars represent mean H/L ratios for the following groups: control (water), low dose (1 X Buctril-M®), and high dose (10 X Buctril-M®). Error bars represent ± SD of the mean.

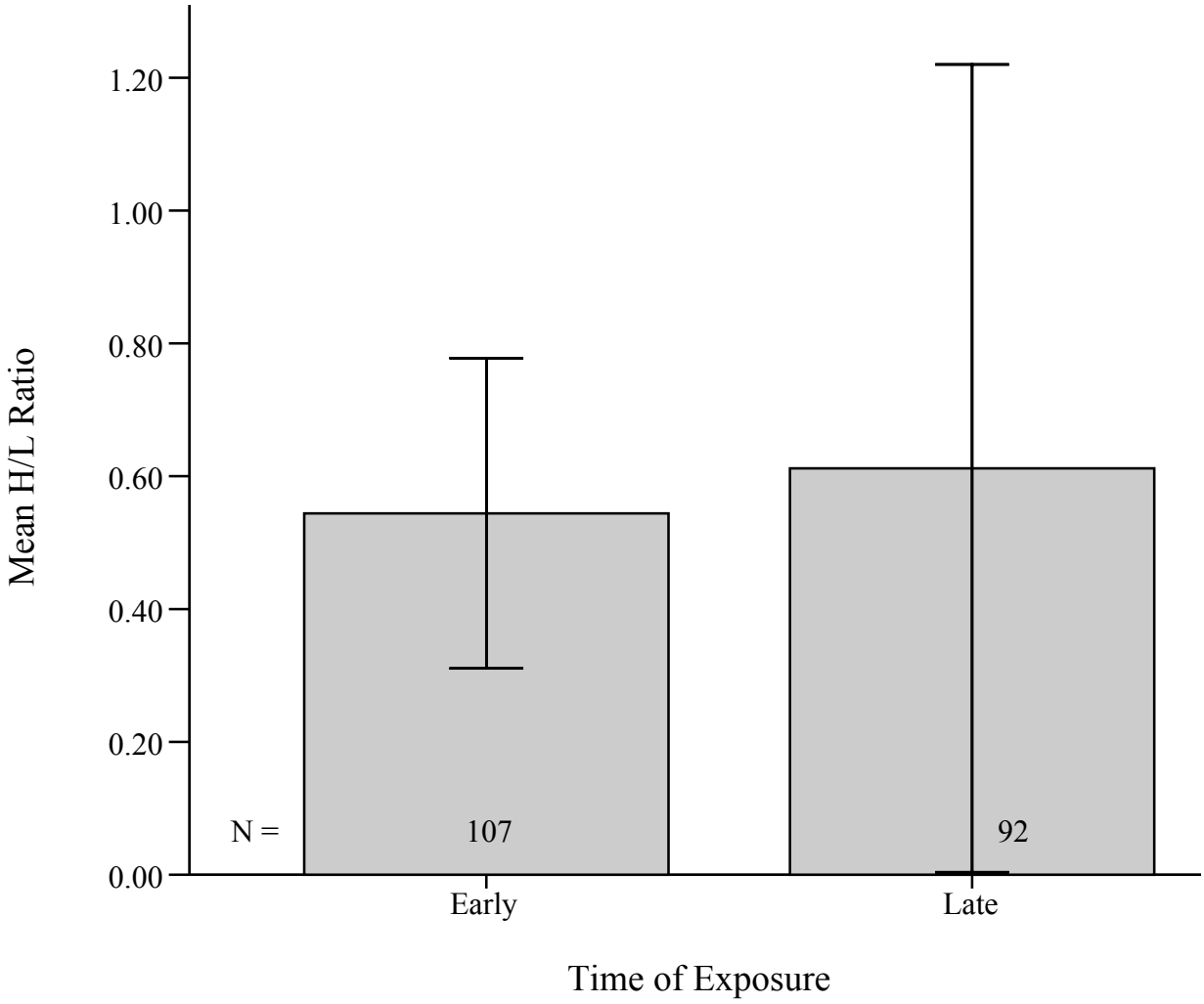

Figure 5-6. The simple association between heterophil/lymphocyte (H/L) ratios in peripheral blood from 21-day-old domestic chickens and the time of *in ovo* Buctril-M® herbicide spray application. The bars represent mean H/L ratios for the following exposure groups: early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

Table 5-7. Descriptive summary of the relative spleen/body weight ratio measured in 21-day-old domestic chickens exposed *in ovo* to Buctril-M® spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	31	0.157	0.037	0.007	0.160	0.130	0.180
	Late[2]	29	0.143	0.028	0.005	0.150	0.130	0.160
1 X Buctril-M® (Low Dose)	Early[1]	30	0.164	0.035	0.006	0.160	0.140	0.180
	Late[2]	34	0.168	0.042	0.007	0.160	0.150	0.180
10 X Buctril-M® (High Dose)	Early[1]	34	0.145	0.034	0.006	0.140	0.128	0.160
	Late[2]	25	0.148	0.031	0.006	0.150	0.135	0.165

[1] Day 6 and [2] Day 15 of incubation

Table 5-8. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the relative spleen/body weight ratio measured in 21-day-old domestic chickens exposed *in ovo* to Buctril-M® formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β		*P* value
		Lower	Upper	
Herbicide Treatment				
- High Dose[1]	-0.003	-0.018	0.012	0.72
- Low Dose[2]	0.018	0.003	0.032	0.02*
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	0.002	-0.012	0.016	0.79
- Late[5]	Reference	-	-	-

[1] 10 X Buctril-M® commercial herbicide formulation spray
[2] 1 X Buctril-M® commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

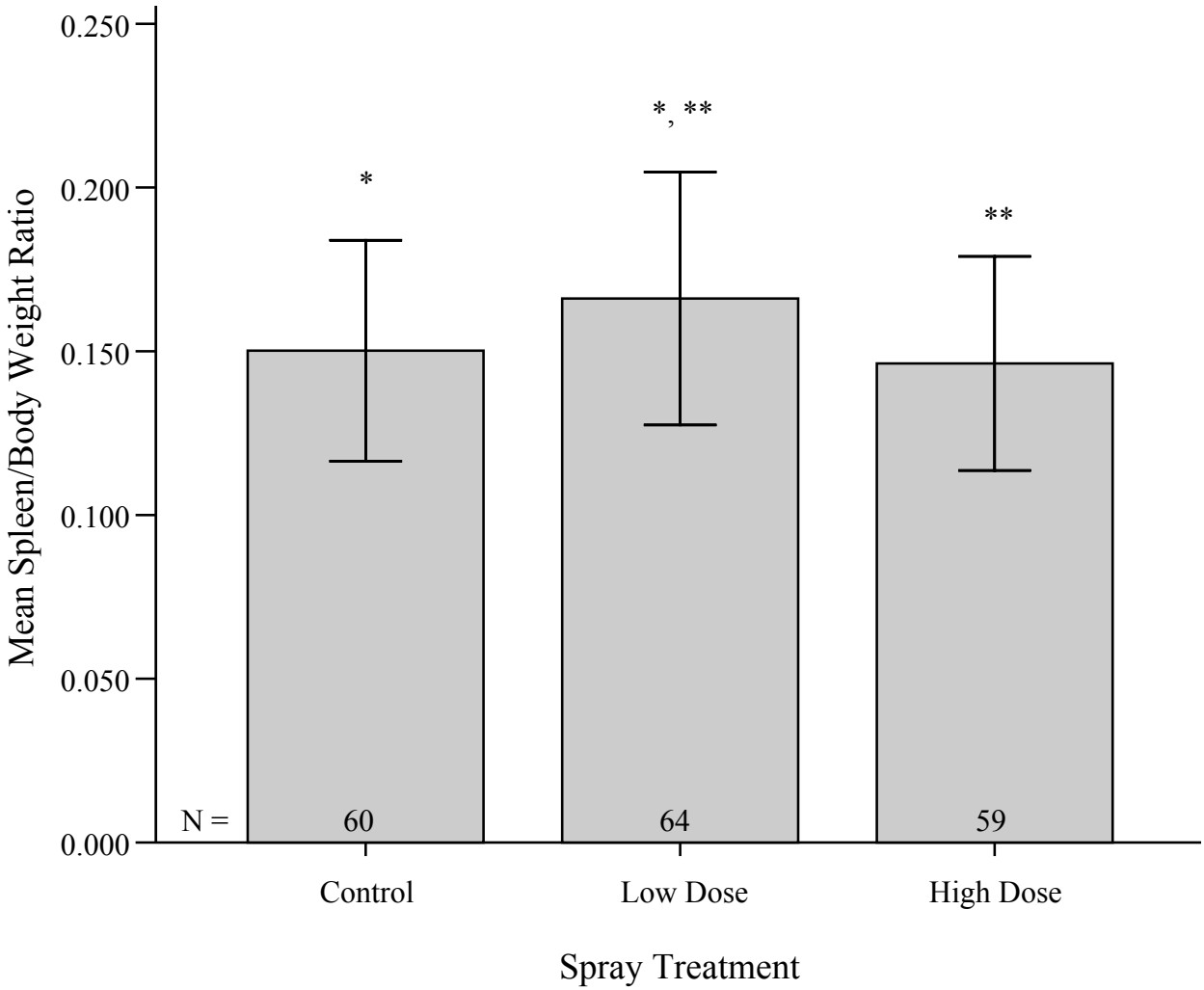

Figure 5-7. The simple association between the mean spleen weight/body weight ratios in 21-day-old domestic chickens and *in ovo* Buctril-M® herbicide exposure. The bars represent the mean relative spleen weights for the following groups: control (water), low dose (1 X Buctril-M®), and high dose (10 X Buctril-M®). Error bars represent ± SD of the mean. *Mean spleen weights/body weights are significantly different between water control and low dose herbicide spray treatments (P = 0.0179). **Mean relative spleen weights are significantly different between the low and high dose herbicide groups (P = 0.0125).

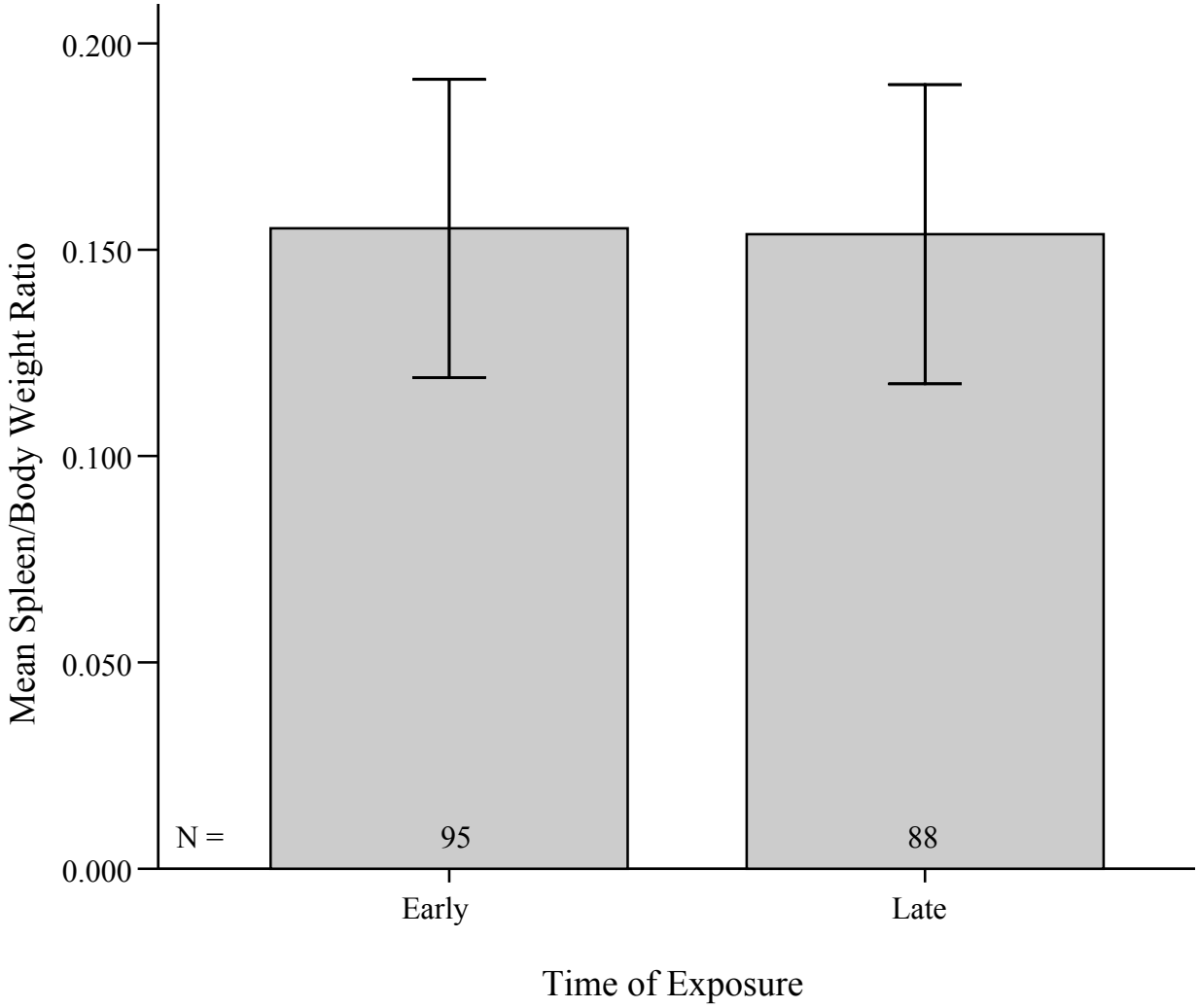

Figure 5-8. The simple association between the mean spleen weight/body weight ratios in 21-day-old domestic chickens and the time of *in ovo* Buctril-M® herbicide spray application. The bars represent the mean relative spleen weights for the following exposure groups: early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

Table 5-9. Descriptive summary of the relative bursa of Fabricius weight/body weight ratio measured in 21-day-old domestic chickens exposed *in ovo* to Buctril-M® spray.

Treatment	Exposure Time	N	Mean	SD	SE	Median	Percentiles 25th	Percentiles 75th
Water (Negative Control)	Early[1]	31	0.475	0.105	0.019	0.460	0.430	0.520
	Late[2]	29	0.473	0.107	0.020	0.500	0.410	0.535
1 X Buctril-M® (Low Dose)	Early[1]	30	0.459	0.110	0.020	0.470	0.358	0.545
	Late[2]	34	0.503	0.111	0.019	0.505	0.445	0.560
10 X Buctril-M® (High Dose)	Early[1]	34	0.437	0.102	0.018	0.460	0.348	0.513
	Late[2]	25	0.435	0.143	0.029	0.420	0.330	0.510

[1] Day 6 and [2] Day 15 of incubation

Table 5-10. Summary of univariate comparisons between the fixed effect factors, exposure time and herbicide treatment, and the relative bursa of Fabricius weight/body weight ratio measured in 21-day-old domestic chickens exposed *in ovo* to Buctril-M® formulation spray.

	Regression coefficient (β)	95% Confidence Intervals for β Lower	95% Confidence Intervals for β Upper	*P* value
Herbicide Treatment				
- High Dose[1]	-0.041	-0.084	0.002	0.06
- Low Dose[2]	-0.002	-0.044	0.040	0.92
- Negative Control[3]	Reference	-	-	-
Time of Exposure				
- Early[4]	-0.007	-0.045	0.031	0.71
- Late[5]	Reference	-	-	-

[1] 10 X Buctril-M® commercial herbicide formulation spray
[2] 1 X Buctril-M® commercial herbicide formulation spray
[3] Water spray
[4] Day 6 of incubation
[5] Day 15 of incubation

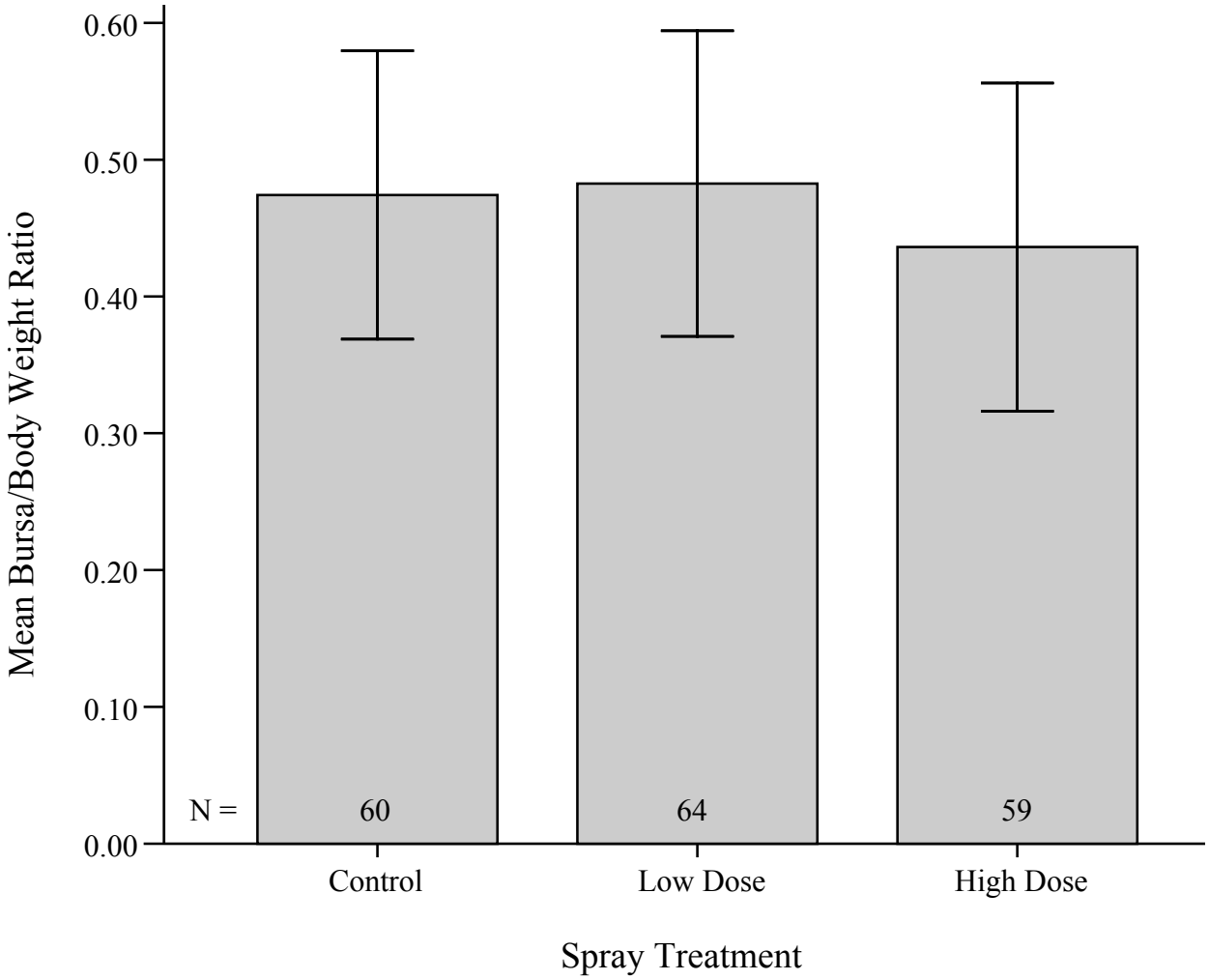

Figure 5-9. The simple association between the mean bursa of Fabricius weight/body weight ratios in 21-day-old domestic chickens and *in ovo* Buctril-M® exposure. The bars represent the mean relative bursa weights for the following groups: control (water), low dose (1 X Buctril-M®), and high dose (10 X Buctril-M®). Error bars represent ± SD of the mean.

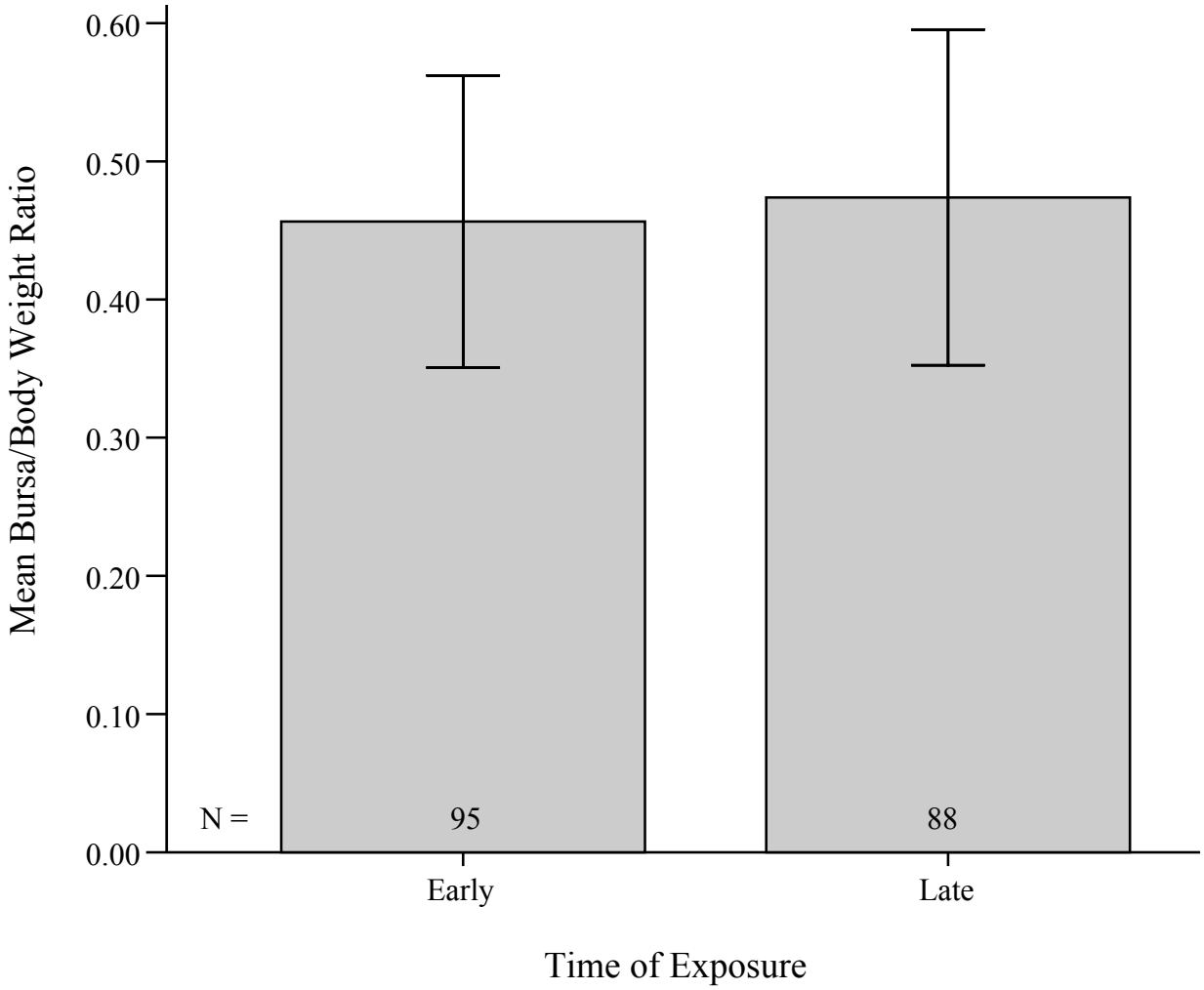

Figure 5-10. The simple association between the mean bursa of Fabricius weight/body weight ratios in 21-day-old domestic chickens and the time of *in ovo* Buctril-M® spray application. The bars represent the mean relative bursa weights for the following exposure groups; early (day 6 of incubation) and late (day 15 of incubation). Error bars represent ± SD of the mean.

5.4 Discussion

The immune system is a structurally complex, highly interactive and balanced system. A competent immune system is essential for the health of an organism, as it reacts rapidly with both non-specific and specific protective responses when the animal is challenged with a foreign substance. Alterations in the immune system include immune modulation expressed as an increase or decrease in measured immune responses, hypersensitivity, and autoimmunity. Derangements of the immune response can put the health of an organism at increased risk from infectious agents, cancers, and other diseases (Blakley and Kouassi 2005). Many anthropogenic compounds have the ability to alter immune function. Therefore, immunomodulating effects of environmental contaminants on specific measures of immune responses can be sensitive biomarkers of toxicant exposure (Keller et al. 2000, Grasman 2002).

Changes in immunocompetence of various wildlife species have been associated with exposure to environmental contaminants. For example, in recent years, studies have investigated the immunotoxic effects of environmental exposure to metals, PCBs, pesticides, and organochlorine compounds in a variety of domesticated and wild birds (Fairbrother and Fowles 1990, Bishop et al. 1998, Smits and Bortolotti 2001, Lee et al. 2001, Lee et al. 2002, Bustnes et al. 2004). Impairment of immunocompetence can result from alterations in the development of the immune system, and these changes may potentially have long lasting, adverse effects on the health of individual animals. If immune impairment is widespread and severe, effects on individual fitness may impact local populations. Since exposure of wildlife to environmental contaminants can occur during the early stages of development, research has recently been directed at evaluating the immunotoxic effects of contaminants during this critical period. In avian species, the period of embryonic development may be particularly vulnerable to contaminant-induced immunomodulation, as the egg may be exposed to xenobiotics through direct contact and transfer through the eggshell. *In ovo* exposure to agrochemicals is a realistic concern for avian species that nest in spring crop cover, because typical spraying periods for early season weed control overlap with the nesting period of many species of upland game birds. Few studies have evaluated the effects of *in ovo* pesticide exposure on the developing immune response in birds (Dabbert et al. 1997, Bishop et al. 1998, Singhal et al. 2003) and significant uncertainty remains around the potential

subtle effects of low-level herbicide exposure during vulnerable stages of embryonic development. The present study attempted to address this research need, by evaluating the effects of a commonly used herbicide on avian immune health and development. A variety of standard immunotoxicity assays were performed to assess potential changes in immune system structure and function in domestic chickens (*Gallus gallus*) following *in ovo* exposure to the commercial herbicide formulation Buctril-M®.

The tests chosen to assess immune function and evaluate potential immunomodulation are standard assays validated by the National Toxicology Program in the USA, and organized into a tiered screening system for suspected immunotoxicants. Assays were chosen from Tiers I and II of the screening system, so that cell-mediated immunity, humoral immune function (antibody production), and general immune system structure were examined in exposed birds. Tier I tests provided a general assessment of immune function and health of associated structural components of the immune system. These tests also included determination of H/L ratios in peripheral blood, measurement of relative lymphoid organ weights (spleen and bursa of Fabricius), and histopathological evaluation of primary and secondary immune organs (thymus, spleen, and bursa of Fabricius) in 21-day-old hatchlings. Possible associations between changes in the immune endpoints of Tier I tests and Buctril-M® exposure were assessed using herbicide treatment (high and low herbicide concentrations) and time of exposure (early and late incubation stages) as fixed effect factors. Differences in these factors did not translate into noticeable effects in the final model analyses for H/L ratios, histopathology results, and relative bursa weights. Relative spleen weight of 21-day-old chicks was associated with the different herbicide treatments (P = 0.01). Mean spleen weight/body weight for birds exposed *in ovo* to the low concentration (recommended field application rate of Buctril-M®) formulated herbicide spray was significantly greater than the mean relative spleen weight of birds in the control group (P = 0.02). Mean relative spleen weights were also significantly different between low dose and high dose herbicide spray treatments (P = 0.01), with relative spleen weights in the high dose group significantly lower than those of the low dose group. However, there was no difference between the high dose group and the controls (P = 0.72), so the reason for the difference in low dose spleen weight is uncertain.

Immunomodulation can be expressed as an enhanced immune response, and result in the increased production of lymphocytes, potentially altering the weight of lymphoid organs. When the pesticide Chlor IPC was tested in the rat, researchers observed an increase in relative spleen weight with concurrent increases in circulating lymphocyte counts (Vos and Kranjc 1983, Vos et al. 1983). However, in the present study, regardless of the higher spleen weights in the low dose group, it is unlikely that this difference is toxicologically significant, because the relative spleen weights for the high dose herbicide group were not significantly different than the control group.

Tier II tests are generally intended to provide a more comprehensive analysis of the immune system, and identify the mechanism of any immune alterations observed in Tier I. Tier II assays used in the present study assessed potential modulation of both the cell- and humoral-mediated immune systems of newly hatched chickens. Humoral immune function was evaluated by measuring the amount of specific antibody produced in response to BSA immunization using an ELISA. Differences in Buctril-M® concentration (fixed effect factor herbicide treatment) and/or timing of Buctril-M® application during incubation (fixed effect factor time of exposure) were not associated with the amount of circulating anti-BSA IgY antibodies in newly hatched chickens. Birds responded as expected to BSA immunization, with serum samples from both 14-day-old chicks (post-BSA primary immunization), and 21-day-old chicks (post-BSA secondary immunization) containing higher concentrations of anti-BSA antibodies (positive reaction against BSA at a higher dilution) than pre-immunization sera. However, there was no association between antibody response and herbicide treatments or the time of herbicide exposure.

The DTH test was used as a specific measure of cell-mediated immune function in newly hatched chickens exposed *in ovo* to Buctril-M®. Birds were sensitized with BSA at both 7 and 14 days of age. At 20 days of age, birds were injected intradermally with BSA in the right wing web. The strength of the DTH reaction was assessed 24 hours later by measuring the change in thickness of the wing web at the injection site, and calculating the difference between pre- and post-injection measurements. Using herbicide treatments (high and low concentrations of Buctril-M® spray) and times of spray exposure (during either early or late stages of incubation) as contributing factors of effect, the DTH response in chickens was found to be statistically similar ($P > 0.05$) among all exposure groups indicating that the

exposure regimes for these birds did not compromise the integrated immune response as measured by the DTH test.

Ground nesting birds are at risk for *in ovo* exposure to agrochemicals. The sublethal effects embryonic exposure to the developing immune system are poorly understood. This study intended to assess the effects of a commonly used herbicide on the immune system of domestic chickens, as a surrogate for wild game birds, when exposed at two distinct timepoints during incubation. The immune system may be particularly vulnerable to toxicant insult during early developmental stages of developing wildlife (Holladay and Smialowicz 2000). Results from most of the tests conducted indicate that *in ovo* exposure to the herbicide Buctril-M® at both the recommended field application concentration (1X) and a concentration representing a worst-case scenario exposure (10X), at the specific incubation stages chosen did not effect the immune endpoints evaluated. The one exception to this general observation was an association between herbicide treatment and relative spleen weight, but the toxicological significance of this finding is unclear, since the size of the spleen may be affected by factors other than the experimental treatments in this study. Future research should be directed at understanding the effects of environmentally relevant herbicide exposures during specific, susceptible periods of avian embryonic growth and development.

CHAPTER 6

GENERAL DISCUSSION

6.1 Research Summary and Fulfillment of Objectives

The overall goal of this research project was to assess the effects of *in ovo* commercial herbicide exposure in newly hatched chickens (*Gallus gallus*) and ducks (*Anas platyrhynchos*). The experimental design was intended to simulate field exposure of wild birds during spring weed control on the Canadian prairies: 1) Domestic chickens and mallard ducks were used as surrogates for wild upland game birds and waterfowl, respectively. As ground nesting species with wide distribution across the prairie pothole region, wild galliformes, ducks and geese are at risk for herbicide exposure during embryonic development *in ovo*. 2) Herbicide spray treatments were applied using an agricultural field spray simulator to reproduce actual field application conditions as closely as possible. 3) Fertile eggs were sprayed with commercial herbicide formulations that are commonly used on cropland in the Canadian prairies. In three separate experiments, chicken eggs were sprayed with a commercial 2,4-D ester product or with Buctril-M® formulation (50:50 mixture of the herbicides bromoxynil and MCPA), and duck eggs were sprayed with the 2,4-D ester formulation. 4) The concentrations of herbicide reflected normal agricultural application rates (low, 1X rate) as well as a potential "worst-case exposure" level (high, 10X rate). 5) In the field, wild bird eggs may be exposed to herbicide spray during any stage of incubation. Developing avian embryos have been shown to be susceptible to a variety of toxicants deposited on and transferred through the egg shell (Lutz and Lutz-Ostertag 1972, Hoffman and Albers 1984, Duffard et al. 1987, Dabbert et al. 1997, Bishop et al. 1998). The degree of sensitivity varies not only with the specific toxicant and dose, but also with the stage of embryonic development. Often the earliest stage of organogenesis is the most highly sensitive (DeWitt et al. 2005), but there are exceptions, depending on the toxicant's target organ and mechanism of action. To account for vulnerability of the embryos at different

stages of development, eggs were exposed to the herbicide during either an early (day 6 for chickens and ducks) or late (day 15 for chickens and day 21 for ducks) incubation stage.

Herbicide deposition on the surface of the eggs was quantified based on results of a fluorescein dye retention study, a technique routinely used to determine the amount of herbicide applied to plant foliage. This approach enabled estimation of the amount of 2,4-D and Buctril-M® each egg received during incubation. In previous studies, herbicide exposure has been quantified by weighing the egg after herbicide application (Hoffman and Albers 1984) or roughly estimated from the herbicide solution prior to spray application (Castro de Cantarini et al. 1989). In the present study 81.7 µg of 2,4-D or 80.8 µg of Buctril-M® active ingredients were deposited on the surface of each chicken egg in the low dose group, while each chicken egg in the 10X application rate group received 789.9 µg of 2,4-D or 785.6 µg of Buctril-M®. Similarly for duck eggs, 2,4-D deposition was calculated to be 87.0 µg and 896.3 µg for each egg in the 1X and 10X application rate groups, respectively.

Few studies have attempted to quantify egg shell penetration of externally applied contaminants. Analysis of 2,4-D residues in chicken and duck eggs in the present study demonstrated measurable transfer of herbicide residues through the shell and into the embryos by 24 hours after spraying. As expected, mean 2,4-D residue concentrations were higher in both chicken and duck eggs from the high dose (10X) groups than in eggs exposed to the recommended field rate of herbicide application (1X). Somewhat unexpectedly, embryo residue concentrations in the chicken eggs (duck eggs not collected) increased from the day following exposure to 5 days after spraying, in both low and high dose groups. Mean concentrations (N = 3) in the 1X group increased from 0.6 to 2.2 ng/g, while 2,4-D residues in the 10X group increased from 27.4 to 374.5 ng/g during this time period. These findings are consistent with previous studies that have demonstrated the transfer of externally applied 2,4-D ester into bird embryos (Somers et al. 1974, Duffard et al. 1987, Castro de Cantarini et al. 1989, Várnagy 1999) and gradual uptake of the herbicide (consequently increasing the amount of compound the embryo is exposed to) over the duration of embryonic development (Castro de Cantarini et al. 1989). The study by Castro de Cantarini et al. (1989) reported that in fertile hen eggs topically exposed to 2,4-D ester on E0, the herbicide was detectable in the embryo by E5, and continued to increase in concentration throughout embryonic

development. These observations suggest that the risk of contaminant-induced adverse effects may continue to increase for at least several days after exposure.

Relatively little is known about the potential long-term effects of low rates of exposure to many herbicides in wild species especially when exposure occurs during embryonic development. Evidence concerning the toxicity of 2,4-D and the active components of Buctril-M®, indicates that these compounds may have subtle effects on genetic material and certain aspects of immune system structure or function. In the present study, potential effects of *in ovo* herbicide exposure on genetic integrity and the immune system of hatchlings was investigated using various biological endpoints. The comet assay and flow cytometry were used to assess induction of DNA strand breaks and chromosomal damage, respectively. Alterations to several aspects of immune function and health were evaluated by determining differential white blood cell counts (specifically the ratio of heterophils to lymphocytes) and immune organ weights, and by the histopathological examination of primary and secondary lymphoid organs. The competence of the specific immune system was assessed using selected immunoassays to evaluate the cell-mediated and humoral immune response of newly hatched birds after immunization with a foreign antigen.

Exposure of fertile chicken and duck eggs to Buctril-M® or 2,4-D had only minor effects on the biomarker of genetic integrity in this study. Differences in herbicide treatment (high and low concentrations) and times of exposure (early and late incubation stages) did not translate into noticeable effects in final model analyses for any of the genotoxicity assay variables evaluated in newly hatched chickens exposed *in ovo* to 2,4-D. Similarly, the comet assay in chicks exposed to Buctril-M® showed that certain measurements of DNA strand breakage were not significantly associated with either herbicide treatment or time of exposure. Results of the comet assay using peripheral lymphocytes from ducklings provided evidence of potential primary genetic damage associated with the time of spray exposure *in ovo*. Comet tail DNA content was significantly associated with spray exposure times such that lymphocytes from 7-day-old ducklings hatched from eggs that were sprayed on day 6 (E6) showed increased amounts of DNA strand breaks compared with cells from birds that were sprayed on day 21 of incubation (E21). This result indicates that ducks may be increasingly sensitive to spray exposure at an early stage of embryological development. This result does not suggest that changes to DNA structure at a earlier timepoint were caused

by the herbicide 2,4-D, as there was no effect of herbicide treatment on DNA strand breakage in ducklings. The damage to DNA in ducklings exposed to spray at E6, observed through one measurement with the comet assay, may be affected by factors other than experimental treatment, and may be influenced by other disturbances to the developing bird as a result of exposing the egg to spray conditions during this sensitive time.

The lack of association between the stage of embryo development (timing of spray exposure) and the genetic changes detected with the comet assay for the chickens may reflect differential sensitivity based on developmental stages. The ducks were sprayed at a relatively earlier embryonic period than the chickens (E6 in ducks represents an earlier developmental stage than E6 in chickens, because of the longer incubation period for ducks). Therefore, duck embryos were treated with spray at an earlier and potentially more vulnerable stage of development. Genetic damage that occurs during early development may be a precursor to specific health problems as an animal matures. Strand breaks that remain unrepaired may lead to permanent genetic mutations, which have been linked to impaired fertility, teratogenesis, or the onset of carcinogenesis or other diseases, depending on the cell type affected (Ponder 2001).

Although the time of embryonic exposure to spray was associated with DNA strand breakage in ducklings, there was no evidence of chromosomal damage. Variation in DNA content (measured by flow cytometry) did not differ significantly between exposure groups, and neither herbicide treatment nor timing of exposure was considered important in the final statistical model. However, in the Buctril-M® experiment, an association between the HPCV values ($\log_{10}$ transformed to attain normality) and time of spray exposure was observed in 21-day-old chickens. The mean HPCV value for the early exposure group (E6) was significantly higher than that of the group treated later in incubation (E15). An increase in HPCV, reflecting greater intercellular DNA variability in chicken erythrocytes, is indicative of increased incidence of chromosomal damage. This genetic effect is considered irreversible (and potentially inheritable), because the DNA content variability interpreted by the flow cytometric measurement HPCV results from early (permanent) clastogenic damage to cell populations (Otto and Oldiges 1980, Deaven 1982, Shugart 1994). It is unclear why early exposure to spray conditions was associated with chromosomal damage, but not with increased occurrence of DNA strand breakage in the comet assay. Possible explanations

include the use of different cell types in the assays, with potential differential sensitivity to genetic damage, or the relatively lower power of the comet assay due to sample size, which may limit the ability to detect some treatment-related effects.

The present study employed a panel of immunotoxicity tests to evaluate the effects of *in ovo* exposure to 2,4-D and Buctril-M® on the developing avian immune system. The tests chosen to assess immune function and evaluate potential immunomodulation in newly hatched birds are standard assays, organized into a tiered screening system for suspected immunotoxicants (Luster et al. 1992, Weeks et al. 1992, Luster et al. 1993, Schuurman et al. 1994). Assays from the Tier I screen performed in this study included differential white blood cell counts, relative immune organ weights and histopathology. Among these variables, H/L ratios and relative immune organ weights demonstrated significant associations with either herbicide treatment or time of exposure in all three experiments. Results of the Tier II functional assays to evaluate effects of *in ovo* exposure on humoral or cell-mediated immune responses in newly hatched chicks and ducklings were not associated with herbicide treatment or exposure time for either 2,4-D or Buctril-M®. Similarly, *in ovo* exposure to both herbicides did not induce structural changes in primary or secondary lymphoid organs, based on histopathological examination of spleen, thymus and bursa in both species.

In 21-day-old chicks exposed *in ovo* to 2,4-D, bursal weight was associated with the different herbicide treatments. Mean bursa weight compared to body weight for birds treated with the low concentration (recommended field application rate) 2,4-D herbicide spray was significantly different from that of birds in the control group. The effect of the high concentration 2,4-D spray on bursal weight compared with controls approached significance. In addition, mean relative bursa weights were significantly different between low dose and high dose herbicide spray treatments. However, while weights were significantly lower in chicks in the low dose group, the opposite effect was observed in the high dose chicks, compared with the controls. The higher bursa weights in the high dose group could represent a compensatory response to increasing herbicide concentration, but with the outcomes from the other immunoassays showing no immunotoxic effect of herbicide treatment, the likelihood that the observed decrease in bursal weight in the low dose birds is causally related to herbicide exposure, or represents a biologically relevant change, is reduced.

The only Tier I test outcome associated with *in ovo* Buctril-M® exposure was the measurement of relative spleen weights in 21-day-old chickens. Herbicide treatment was found to be an important factor in the initial analysis, influencing differences between groups in the final univariate comparison model. Relative spleen weights for birds in the low dose treatment groups were significantly different than both the control and high dose groups. In the experiments using newly hatched chickens as the animal model, the association between relative spleen weights and Buctril-M® treatment was comparable to the association observed between relative bursa weights and 2,4-D treatment. In both cases, birds that were treated with a low concentration of herbicide demonstrated different weights than both the control and high dose groups. However, there was no significant difference between high dose and control groups, so the results were not considered biologically or toxicologically significant.

Relative bursa weight was associated with time of spray exposure in the study involving *in ovo* 2,4-D exposure of duck eggs. The mean relative weight of the bursa of Fabricius from 21-day-old ducklings exposed to 2,4-D on day 6 of incubation (E6) was greater than the mean relative weight of birds from the groups exposed on day 21 (E21). Herbicide treatment was not associated with differences in bursal weight, so the former observation does not imply that 2,4-D treatment at different times during incubation affected bursal weight. Spray exposure during earlier stages of development may affect the humoral immune response, if increased bursal weight is associated with increased production of precursor B cells that provide the humoral response.

Total and differential blood cell counts are important variables in the assessment of the health of an organism. In avian immunotoxicity studies, differential white blood cells counts are routinely used as a general immune health indicator, and the ratio of heterophils (avian granulocytes instrumental in the non-specific defense response, due to their large numbers and ability to phagocytize foreign bodies and bacteria, equivalent to the mammalian neutrophil) to lymphocytes (mononuclear white blood cells responsible for the recognition and destruction of many types of pathogens) is used as a measurement of stress. Mean H/L ratios in blood from 21-day-old ducklings exposed *in ovo* to 2,4-D were significantly different between the groups treated with the high concentration of 2,4-D and water (control). Although ratios from the birds in the low dose groups were not significantly different from the control groups, changes in H/L ratio values demonstrated a dose dependent trend with

increasing herbicide exposure. Previous studies have demonstrated that exposure to various types of stressors, including environmental contaminants, results in increasing numbers of heterophils in peripheral circulation, and an upward shift in the H/L ratio in birds (Maxwell and Robertson 1998).

In this study, relatively few of the outcomes from the genotoxicity and immune function assays were associated with herbicide exposure in the final analysis. Differences in herbicide treatment (low and high herbicide concentrations), for either 2,4-D or Buctril-M® formulation, did not result in changes to experiment outcomes that were of toxicological significance and posed a risk to the health and development of newly hatched birds. Timing of spray exposure (although not directly related to herbicide treatment) proved to be an important fixed effect factor for the majority of the significant associations observed and therefore warrants further discussion.

Critical stages of development during which vertebrate species are most vulnerable to the toxic effects of environmental contaminants include embryogenesis and the neonatal or early post hatching period (Hoffman 1990b). Toxic effects that occur during early life stages have the potential to cause alterations at the lower levels of biological organization in developing systems, and subtle changes in genes, cells, tissues, body chemical processes and functions occur before more severe disturbances are observable (or even measurable) at the population and ecosystem level. Biochemical and molecular effects can be detected as changes in enzyme levels, in structure of cell membranes, and in genetic material, or DNA (Shugart 1992). Specific types of changes to genetic integrity (clastogenic alterations, DNA adducts, strand breaks, etc.) can be used as endpoints for assessing exposure to genotoxicants. Persistence of genetic damage may induce a series of structural and functional (potentially deleterious) responses at higher levels of biological organization (Shugart 1999).

Complex processes such as hormonal regulation, metabolism, and immune system responses can be impaired by subtle changes incurred during early stages of development. The potential for persistent deleterious effects on wildlife health following early, low-level contaminant exposure has gained recent attention in part because the developing immune system has been identified as a particularly sensitive target for chemically-induced immunomodulation (Holladay and Smialowicz 2000). Potential adverse effects caused by

exposure to toxic agents during development may range from damage to vital (structural and functional) components of the immune system, to altered or poorly regulated immune responses. These effects may potentially impact the organism's ability to compete and reproduce.

The potential vulnerability of the developing avian embryo to environmental contaminant exposure *in ovo* has been investigated in a number of experiments. Results from several studies suggest that the embryonic day of contaminant exposure is an important factor of effect, and influences the type and severity of adverse impacts to developing systems (Lee et al. 2001, Lee et al. 2002, DeWitt et al. 2005, Heinz et al. 2006). Up to embryonic day 4 is the primary period of organogenesis, when initial organ development begins. In chickens, E6 represents a relatively late stage of organogenesis, while E15 coincides with a period of later differentiation (Patten 1971, Romanoff and Romanoff 1972). Development in ducks is somewhat slowed because of the longer incubation period. Because vulnerability is time-specific, it is important to expose the embryo to contaminant throughout incubation to adequately assess potential toxic effects on avian development.

In agricultural areas of the Canadian prairies, wild bird eggs have the potential to be exposed to herbicide at any time after they are laid. Consequently, in order to increase environmental applicability, the present study evaluated embryo sensitivity at two distinct exposure times during incubation. Differences in times of herbicide exposure proved to be an important factor of effect for outcomes from both the genotoxicity assays (comet assay and flow cytometric DNA analysis) and the general tests for potential immunomodulation (H/L ratio and relative immune organ weight) in all experiments.

Although subtle changes to the genetic integrity and immune system components of newly hatched birds were demonstrated after *in ovo* herbicide exposure in the present study, earlier, more vulnerable stages of avian development may have been missed. Given the possibility of adverse consequences of these effects on the health of developing birds, further research is recommended in order to adequately assess the risks of 2,4-D and Buctril-M®, as well as other widely used herbicides and agrochemicals. The lack of significant adverse effects observed following *in ovo* exposure in this study represents good news for avian wildlife on the Canadian prairies, but with millions of eggs of numerous species likely to be exposed every spring, there are many questions that remain unanswered.

CHAPTER 6

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